Urinary markers of glomerular and tubular damage in chronic kidney disease

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URINARY MARKERS OF GLOMERULAR AND TUBULAR DAMAGE IN CHRONIC KIDNEY DISEASE

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
woensdag 18 januari 2012
om 16.15 uur

door

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geboren op 10 december 1981
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Financial support was kindly provided by:

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Illustration on cover is extracted from Trousset encyclopedia (1886-1891)

Printed by: Wöhrmann

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General introduction and aims of the thesis

Published in revised form in:

Current Opinion in Nephrology and Hypertension. 19: 513-518, 2010

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Introduction

The diagnosis of chronic kidney disease (CKD) is based on two criteria: the presence of impaired kidney function, expressed as glomerular filtration rate less than 60 ml/min/1.73m^2, and signs of kidney damage, usually expressed as urinary albumin excretion more than 30 mg/day. In the last decade many studies have assessed to what extent CKD predicts outcomes, not only in terms of all-cause and cardiovascular mortality but also in terms of renal prognosis, that is, incident end-stage renal disease (ESRD) and/or progression of CKD.In this chapter it will be discussed, first, what the available evidence is that albuminuria is a predictor of both cardiovascular and renal prognosis independent of the level of estimated
glomerular filtration rate (eGFR). Second, it will be described why albuminuria predicts these outcomes independently of eGFR. Third, it will evaluated whether risk prediction can be further improved by measuring manifestations of renal damage other than albuminuria and eGFR. Therefore, the question whether albuminuria is all you need to predict outcomes in CKD or that specific tubular damage markers can further improve our predictive capacity for outcomes in CKD will be addressed. Lastly, a brief introduction to the various chapters of this thesis will be given.

Albuminuria and glomerular filtration rate to predict cardiovascular outcome

Although it has been appreciated for many years that patients with end-stage renal disease (CKD stage 5) have a worse cardiovascular survival than age and sex-matched healthy controls, it was established only relatively recently that patients with mildly impaired kidney function (eGFR <60 ml/min/ 1.73m2, CKD stage 3-4)) already have a worse cardiovascular prognosis. [1-7]
The association of albuminuria with cardiovascular outcomes has also been described in other studies. Although it has long been known that elevated albuminuria predicts worse cardiovascular survival in individuals with diabetes[8], in the last decade similar associations have been observed in individuals with hypertension[9], the elderly[10], and in the general population.[11]

In a recent meta-analysis the 'dose dependency' of this association was shown: microalbuminuria was associated with a 47% increased risk for cardiovascular events and macroalbuminuria with a 117% increased risk.[12] Elevated albuminuria and impaired eGFR frequently coincide. It is, therefore, important to study the interrelationship between albuminuria and impaired eGFR for predicting poor cardiovascular survival. This was done in a recent meta-analysis by the CKD Prognosis Consortium, in which it was shown that albuminuria and eGFR independently of each other – and independently of traditional cardiovascular risk factors – are associated with all-cause and cardiovascular mortality.[13]

Albuminuria and glomerular filtration rate to predict renal outcome

Most of the data on the predictive value of albuminuria and GFR for kidney outcomes have been obtained from studies that included patients with known kidney disease in an advanced stage and/or macroalbuminuria.[14-16] Only recently, data on kidney outcomes in cohorts based on the general population have become available.[17-20] In these studies the authors evaluated the need to start renal replacement therapy. The data show that the incidence of starting renal replacement therapy is increased not only for lower baseline eGFR, but also for higher baseline albuminuria. An increased risk for renal replacement therapy was manifest already in the microalbuminuria range.[17-20]

Although all studies agreed upon the fact that low eGFR is associated with an increased risk for incident CKD, only few evaluated whether the same is true for albuminuria.[18;20;21] These latter studies showed, similar to studies with ESRD as outcome, that albuminuria (and dipstick proteinuria) predict progressive CKD as well as acute kidney injury independent of the level of eGFR.

Why do albuminuria and glomerular filtration rate predict renal and vascular outcomes independently of each other?

As it has become clear that eGFR and albuminuria, independently of each other and of traditional cardiovascular risk factors, predict both cardiovascular mortality and kidney outcomes, the question arises of why these two kidney-derived parameters add to each other's predictive performance. Of course, impaired renal clearance and leakage of albumin through the glomerular filter are separate phenomena. It may well be that the former contributes in a different way to vascular and renal prognosis than the latter. Leakage of albumin from the circulation into urine is still frequently considered merely a glomerular representation of the presence and severity of systemic endothelial dysfunction.[22;23] Once albumin is filtered by the glomerulus the majority is reabsorbed from the ultrafiltrate by proximal tubular epithelial cells.[24] Although eGFR reflects glomerular function, albuminuria may well be more a reflection of tubular than of glomerular function. As tubulointerstitial damage on histological examination is better associated with kidney function than glomerular damage in various kidney diseases, for example in diabetic nephropathy[25], the association of albuminuria with tubular dysfunction may explain why albuminuria offers additive precision to predict renal prognosis. Given these considerations, why then does albuminuria add to GFR to predict cardiovascular prognosis? As the loss of albumin in urine is unlikely to be causally related to a worse cardiovascular outcome, it seems likely that albuminuria reflects another underlying phenomenon resulting in vascular events. The STENO hypothesis states that albuminuria is a reflection of endothelial damage.[23] As such it relates to a generalized endothelial abnormality and is not restricted to endothelial damage in the kidney alone. It has been argued that albuminuria (especially in the microalbuminuria range) is just a marker of damage related to generalized atherosclerosis that also affects the kidneys.[26] This is in line with the tubular hypoxia hypothesis[27], that generalized atherosclerosis may impair blood flow in peritubular capillaries more than it impairs overall renal blood flow. Such an impaired flow in the peritubular

capillaries may result in tubulointerstitial damage and interstitial fibrosis causing impaired oxygen diffusion and supply to tubular cells, resulting in impaired tubular albumin reabsorption. This may explain why albuminuria is closely related to both renal and vascular prognosis.

Can risk prediction by albuminuria and glomerular filtration rate be further improved?

In the PREVEND study the value of albuminuria and eGFR in addition to other cardiovascular risk factors to predict renal outcome has been investigated. The best predictive model included not only age, systolic blood pressure, C-reactive protein and known hypertension, but also eGFR and albuminuria,[28] indicating an important independent predictive role for these renal parameters that is stronger than many other more conventional predictors of renal outcome. Another question to answer is whether other parameters, beyond the classical cardiovascular risk factors on the one hand and eGFR and albuminuria on the other, could further improve the predictive vale of renal risk scores. Previously, it has been shown in individuals with primary renal diseases that high-molecular-weight proteins[29] and in particular urinary IgG excretion[30] predict renal prognosis better than overall proteinuria. This may be true for individuals with overt proteinuria, in which the majority of the protein (>70%) is albumin and only a small fraction is composed of high-molecular-weight proteins. In individuals with lower amounts of total urinary protein, however, that is in individuals who excrete less than 500mg of proteins per day, a relatively small fraction of the protein (e.g. less than 50%) consists of albumin and the remainder consists of lower molecular weight proteins that are handled by the tubules. Whether the measurement of the urinary excretion of these tubular proteins may add to the predictive capacity of albumin for kidney outcomes is currently being investigated. For example, it has recently been shown that albuminuria and nonalbumin urinary protein excretion predict graft loss after renal transplantation in an additive manner.[31]

Can specific tubular markers add to the predictive value of albuminuria?

In recent years, specific damage markers of the proximal and distal tubule have been discovered. They have been extensively investigated in the field of acute kidney injury, and more recent, also in chronic kidney disease. In this thesis, several markers of the glomerulus, proximal tubule, distal tubule and general inflammation were measured, both in urine as in plasma. Urinary markers of renal injury are elevated during glomerular and tubulointerstitial injury and provide a non-invasive tool to monitor renal damage.

In this thesis IgG and IgG-4 were measured as glomerular markers. Urinary loss of the positively charged IgG (150 kDa) is associated with loss of size selectivity of the glomerular basement membrane, whereas urinary loss of the negatively IgG-4 is considered to reflect loss of charge selectivity.[32] Urinary IgG predicts the onset of end-stage-renal-disease in CKD patients even better than albuminuria. The fractional excretion of IgG is superior to proteinuria in predicting progression of CKD.

The tubular injury markers investigated here reflect injury in different renal compartments and are mediated by different processes. KIM-1, NAG, Cystatine C and ß-2-microglobuline were measured as markers for proximal tubular damage. KIM-1 is selectively expressed by injured proximal tubular cells.[33] KIM-1 expression is related to tubulointerstitial damage and correlates with the severity of kidney function impairment.[34] Urinary excretion of KIM-1 predicts the onset of end stage renal disease.[35]

NAG (135 kDa) is a lysosymal enzyme that is predominantly produced in the proximal tubule, and released into urine upon cellular damage. Elevated urinary NAG is elevated in hypertensive subjects, predicts the subsequent occurrence of albuminuria in diabetic patients, and was found to predict CKD progression better than proteinuria in non-diabetic CKD.

Cystatin C is an endogenous cysteine proteinase inhibitor, produced by most nucleated cells at a relatively constant rate and released into the plasma. In normal renal function, tubular reabsorption and catabolism of cystatin C is almost complete and cystatin C is thus only detectable in very small quantities in the urine.[36] Little is yet known about the predictive value of urinary cystatin C in chronic kidney disease, but in diabetes it is cross-sectionally associated with eGFR and albuminuria.[37]
β-2-microglobulin (12 kDa) is a component of MHC class 1 molecules, which are present on all nucleated cells. β-2-microglobulin is freely filtered through the glomerulus and subsequently reabsorbed by proximal tubular cells. Urinary β-2-microglobulin is a marker of proximal tubular reabsorption incapacity and predicts the rate of CKD progression.[38-40]

H-FABP was measured as marker of distal tubular damage. H-FABP (15kDa) is an intracellular carrier protein that is present in cytoplasm of distal tubular cells. Urinary H-FABP results from release by structurally damaged tubular cells.[41;42] Elevated urinary H-FABP predicts prognosis in CKD.[43]

NGAL and MCP-1 were measured as markers of general inflammation. NGAL (25 kDa) is expressed by neutrophils and other epithelial cells. It was found to reflect damage to glomeruli, and proximal and distal tubules.[44-46] Elevated urinary NGAL predicts CKD progression.[47]

MCP-1 (13-30 kDa) is expressed by inflammatory cells such as monocytes, and also by resident renal cells, i.e. mesangial, endothelial, and tubular epithelial cells.[48] Renal cells produce MCP-1 in response to a variety of pro-inflammatory stimuli.[49] Elevated urinary MCP-1 predicts the rate of renal function loss in CKD.[50]

Consequences?

Despite all the evidence that albuminuria is an important and independent predictor of cardiovascular and renal outcomes in a diversity of populations, including the general population, the question whether albuminuria is all you need to predict outcomes in CKD, should be answered with a 'no'. To reliably predict outcomes one should, of course, also have information on other parameters, including eGFR, blood pressure, diabetes status and underlying renal disease. The fact that albuminuria predicts cardiovascular and kidney outcomes independent of baseline eGFR implies that measurement of albuminuria should at least be part of screening programs for CKD. It is argued that measurement of albuminuria could be used as a first step in screening programs to identify high-risk CKD individuals instead of measurement of eGFR. It may well be that albuminuria adds to the predictive value of eGFR because albuminuria is not only related to glomerular damage, but also relates to tubular damage. As such it would be interesting to study whether (other) tubular markers will further improve the ability of albuminuria and eGFR to predict outcomes in CKD.

Aims of the thesis

The general aim of this thesis was to explore whether measuring the aforementioned renal damage markers may have value in addition to measuring the at present clinically used damage marker, albuminuria. Several purposes of use can be thought of. For instance, these markers could be of help in identifying patients with early tubulo-interstitial damage. Second, these markers could be useful in predicting renal prognosis. Third, they could potentially be used as markers to monitor the effect of medication.

Outline of the thesis

In **chapter 2** damage markers of glomerular and tubular function were measured in a cohort of patients with diabetes mellitus. First, it was investigated how these damage markers correlate to renal damage, assessed as eGFR and albuminuria. A significant correlation between

damage marker and eGFR and albuminuria indicates that a marker is promising to study progression of renal disease. Second, it was investigated whether these damage markers are already elevated before albuminuria starts to rise. This would suggest that these markers are very early and sensitive markers of renal damage. Finally, we looked whether the damage markers under investigation are independently of albuminuria associated with GFR, since this might indicate whether these markers will add to the predictive capacity of albuminuria.

Importantly, all damage markers were measured in fresh urine samples. In epidemiological research, however, most studies use samples that have been stored frozen during prolonged periods of time. Previously it has been shown that frozen storage during 1 year is associated with a decrease in urinary albumin concentration, with a wide variation between urine samples in the amount to which albumin decreases.[51;52] These storage effects are of importance, as is has been shown that the value of urinary albumin excretion to predict mortality is considerably better when assessed in fresh urine samples in comparison to when assessed in the same samples after prolonged frozen storage.[53] Therefore in **chapter 3** it is investigated whether alkalinisation of urine samples before freezing preserves albumin concentration. We stored unadjusted and alkalized urine samples of patients with diabetes mellitus for 1 year at -20º C and at -80º C. After 1 year of frozen storage we measured albumin concentration again.

Since we planned to further investigate whether specific tubular damage markers add to the predictive capacity of albuminuria, it was examined whether these markers can reliably be measured from urine samples that have been frozen stored for prolonged periods of time. Therefore we investigated in **chapter 4** the effect of frozen storage on urinary renal damage markers. First we measured urinary damage markers fresh, after 1 week, after 6 months and after 1 year of frozen storage to see whether a potential decline will stabilize. Second, it was similarly as in chapter 3

investigated whether the effect of 1 year frozen storage at -80º C and -20º C can be influenced by different storage protocols.

As discussed in chapter 2 it may be that urinary damage markers predict renal outcome in addition to albuminuria. Therefore it is investigated in **chapter 5** whether specific damage markers predict renal outcome defined as progression of renal function loss or need for renal replacement therapy in a cohort of 606 renal transplant recipients. In this cohort with a median follow-up of almost 5 years we compared different urinary damage markers to proteinuria and albuminuria, the two as yet in clinical practice most used markers for risk prediction. It was examined whether these markers add to the predictive capacity of proteinuria or albuminuria in predicting graft failure and renal function loss.

Progression of CKD can be assessed as a decrease in GFR (as investigated in chapter 5) or as an increase in albuminuria. The latter is a relevant outcome since it has been shown that a rise in albuminuria is associated with a worse cardiovascular[54] and renal prognosis.[55] In **chapter 6** therefore the value of these markers to predict outcome defined as progression of albuminuria during follow-up is investigated in the PREVEND cohort. This cohort of 8592 participants is selected from the general population. They are followed over time during serial screenings, with an average follow-up of 9.3 years.
Besides predicting outcome, tubular damage markers may also be of help to monitor the effect of treatment on short term basis. That is why in **chapter 7** the effect of various antiproteinuric regimens (i.c. dietary sodium restriction and addition of an angiotensin II receptor blocker (ARB)) on renal damage markers is investigated in 52 non-diabetic patients with chronic kidney disease. We investigated whether these antiproteinuric interventions are also followed by decrease in renal damage markers. Furthermore we looked whether intensified reduction of proteinuria to levels below 0.3 gram/day by

combinations of ACE inhibition, ARB and dietary sodium restriction, is accompanied by normalisation of tubular damage markers in these patients.

Chapter 8 summarizes all studies performed, integrates them into current literature and tries to answer the question whether assessment of urinary damage markers in addition to measuring albuminuria may be of help in clinical practice.

Reference List

1. Weiner DE, Tighiouart H, Amin MG *et al*. Chronic kidney disease as a risk factor for cardiovascular disease and all-cause mortality: a pooled analysis of community-based studies. *J Am Soc Nephrol* 2004; 15: 1307-1315

2. Shlipak MG, Simon JA, Grady D, Lin F, Wenger NK, Furberg CD. Renal insufficiency and cardiovascular events in postmenopausal women with coronary heart disease. *J Am Coll Cardiol* 2001; 38: 705-711

3. Shlipak MG, Heidenreich PA, Noguchi H, Chertow GM, Browner WS, McClellan MB. Association of renal insufficiency with treatment and outcomes after myocardial infarction in elderly patients. *Ann Intern Med* 2002; 137: 555-562

4. Shlipak MG, Sarnak MJ, Katz R *et al*. Cystatin C and the risk of death and cardiovascular events among elderly persons. *N Engl J Med* 2005; 352: 2049-2060

5. Shlipak MG, Fried LF, Cushman M *et al*. Cardiovascular mortality risk in chronic kidney disease: comparison of traditional and novel risk factors. *JAMA* 2005; 293: 1737-1745

6. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med* 2004; 351: 1296-1305

7. Muntner P, He J, Hamm L, Loria C, Whelton PK. Renal insufficiency and subsequent death resulting from cardiovascular disease in the United States. *J Am Soc Nephrol* 2002; 13: 745-753

8. Mogensen CE. Microalbuminuria predicts clinical proteinuria and early mortality in maturity-onset diabetes. *N Engl J Med* 1984; 310: 356-360

9. Wachtell K, Ibsen H, Olsen MH *et al*. Albuminuria and cardiovascular risk in hypertensive patients with left ventricular hypertrophy: the LIFE study. *Ann Intern Med* 2003; 139: 901-906

10. Damsgaard EM, Froland A, Jorgensen OD, Mogensen CE. Microalbuminuria as predictor of increased mortality in elderly people. *BMJ* 1990; 300: 297-300

11. Hillege HL, Fidler V, Diercks GF *et al*. Urinary albumin excretion predicts cardiovascular and noncardiovascular mortality in general population. *Circulation* 2002; 106: 1777-1782

12. Perkovic V, Verdon C, Ninomiya T *et al*. The relationship between proteinuria and coronary risk: a systematic review and meta-analysis. *PLoS Med* 2008; 5: e207

13. Matsushita K, van der Velde M, Astor BC *et al*. Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. *Lancet* 2010; 375: 2073-2081

14. Ruggenenti P, Perna A, Mosconi L, Pisoni R, Remuzzi G. Urinary protein excretion rate is the best independent predictor of ESRF in non-diabetic proteinuric chronic nephropathies. "Gruppo Italiano di Studi Epidemiologici in Nefrologia" (GISEN). *Kidney Int* 1998; 53: 1209-1216

15. Jafar TH, Stark PC, Schmid CH *et al*. Progression of chronic kidney disease: the role of blood pressure control, proteinuria, and angiotensin-converting enzyme inhibition: a patient-level meta-analysis. *Ann Intern Med* 2003; 139: 244-252

16. Keane WF, Zhang Z, Lyle PA *et al*. Risk scores for predicting outcomes in patients with type 2 diabetes and nephropathy: the RENAAL study. *Clin J Am Soc Nephrol* 2006; 1: 761-767

17. Iseki K, Kinjo K, Iseki C, Takishita S. Relationship between predicted creatinine clearance and proteinuria and the risk of developing ESRD in Okinawa, Japan. *Am J Kidney Dis* 2004; 44: 806-814

18. van der Velde M, Halbesma N, de Charro FT *et al*. Screening for albuminuria identifies individuals at increased renal risk. *J Am Soc Nephrol* 2009; 20: 852-862

19. Hallan SI, Ritz E, Lydersen S, Romundstad S, Kvenild K, Orth SR. Combining GFR and albuminuria to classify CKD improves prediction of ESRD. *J Am Soc Nephrol* 2009; 20: 1069-1077

20. Hemmelgarn BR, Manns BJ, Lloyd A *et al*. Relation between kidney function, proteinuria, and adverse outcomes. *JAMA* 2010; 303: 423-429

21. Verhave JC, Gansevoort RT, Hillege HL, Bakker SJ, de Zeeuw D, de Jong PE. An elevated urinary albumin excretion predicts de novo development of renal function impairment in the general population. *Kidney Int Suppl* 2004; S18-S21

22. Bakker SJ, Gansevoort RT, Stuveling EM, Gans RO, de Zeeuw D. Microalbuminuria and C-reactive protein: similar messengers of cardiovascular risk? *Curr Hypertens Rep* 2005; 7: 379-384

23. Deckert T, Feldt-Rasmussen B, Borch-Johnsen K, Jensen T, Kofoed-Enevoldsen A. Albuminuria reflects widespread vascular damage. The Steno hypothesis. *Diabetologia* 1989; 32: 219-226

24. Bakker SJ, Gansevoort RT, de Zeeuw D. Albuminuria: what can we expect from the determination of nonimmunoreactive albumin? *Curr Hypertens Rep* 2009; 11: 111-117

25. Dalla Vestra M, Saller A, Bortoloso E, Mauer M, Fioretto P. Structural involvement in type 1 and type 2 diabetic nephropathy. *Diabetes Metab* 2000; 26 Suppl 4: 8-14

26. El Nahas M. Cardio-Kidney-Damage: a unifying concept. *Kidney Int* 2010; 78: 14-18

27. Nangaku M. Chronic hypoxia and tubulointerstitial injury: a final common pathway to end-stage renal failure. *J Am Soc Nephrol* 2006; 17: 17-25

28. Halbesma N, Jansen DF, Heymans MW, Stolk RP, de Jong PE, Gansevoort RT. Development and Validation of a General Population Renal Risk Score. *Clin J Am Soc Nephrol* 2011; 6: 1731-1738

29. Mackinnon B, Shakerdi L, Deighan CJ, Fox JG, O'Reilly DS, Boulton-Jones M. Urinary transferrin, high molecular weight proteinuria and the progression of renal disease. *Clin Nephrol* 2003; 59: 252-258

30. Reichert LJ, Koene RA, Wetzels JF. Urinary IgG excretion as a prognostic factor in idiopathic membranous nephropathy. *Clin Nephrol* 1997; 48: 79-84

31. Halimi JM, Matthias B, Al Najjar A *et al*. Respective predictive role of urinary albumin excretion and nonalbumin proteinuria on graft loss and death in renal transplant recipients. *Am J Transplant* 2007; 7: 2775-2781

32. Hemmelder MH, de Zeeuw D, de Jong PE. Measurement of glomerular charge selectivity in non-diabetic renal disease. *Nephrol Dial Transplant* 1997; 12 Suppl 2: 57-62

33. Waanders F, Navis G, van Goor H. Urinary tubular biomarkers of kidney damage: potential value in clinical practice. *Am J Kidney Dis* 2010; 55: 813-816

34. Waanders F, van Timmeren MM, Stegeman CA, Bakker SJ, van Goor H. Kidney injury molecule-1 in renal disease. *J Pathol* 2010; 220: 7-16

35. Peters HP, Waanders F, Meijer E *et al*. High urinary excretion of kidney injury molecule-1 is an independent predictor of end-stage renal disease in patients with IgA nephropathy. *Nephrol Dial Transplant* 2011;

36. Herget-Rosenthal S, Feldkamp T, Volbracht L, Kribben A. Measurement of urinary cystatin C by particle-enhanced nephelometric immunoassay: precision, interferences, stability and reference range. *Ann Clin Biochem* 2004; 41: 111-118

37. Nauta FL, Boertien WE, Bakker SJ *et al*. Glomerular and tubular damage markers are elevated in patients with diabetes. *Diabetes Care* 2011; 34: 975-981

38. Drueke TB, Massy ZA. Beta2-microglobulin. *Semin Dial* 2009; 22: 378-380

39. Gerritsen KG, Peters HP, Nguyen TQ *et al*. Renal proximal tubular dysfunction is a major determinant of urinary connective tissue growth factor excretion. *Am J Physiol Renal Physiol* 2010; 298: F1457-F1464

40. Branten AJ, du Buf-Vereijken PW, Klasen IS *et al*. Urinary excretion of beta2-microglobulin and IgG predict prognosis in idiopathic membranous nephropathy: a validation study. *J Am Soc Nephrol* 2005; 16: 169-174

41. Maatman RG, Van Kuppevelt TH, Veerkamp JH. Two types of fatty acid-binding protein in human kidney. Isolation, characterization and localization. *Biochem J* 1991; 273 (Pt 3): 759-766

42. Pelsers MM. Fatty acid-binding protein as marker for renal injury. *Scand J Clin Lab Invest Suppl* 2008; 241: 73-77

43. Hofstra JM, Deegens JK, Steenbergen EJ, Wetzels JF. Urinary excretion of fatty acid-binding proteins in idiopathic membranous nephropathy. *Nephrol Dial Transplant* 2008; 23: 3160-3165

44. Mishra J, Ma Q, Prada A *et al*. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *J Am Soc Nephrol* 2003; 14: 2534-2543

45. Kuwabara T, Mori K, Mukoyama M *et al*. Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons. *Kidney Int* 2009; 75: 285-294

46. Bonventre JV, Vaidya VS, Schmouder R, Feig P, Dieterle F. Next-generation biomarkers for detecting kidney toxicity. *Nat Biotechnol* 2010; 28: 436-440

47. Bolignano D, Lacquaniti A, Coppolino G *et al*. Neutrophil gelatinase-associated lipocalin (NGAL) and progression of chronic kidney disease. *Clin J Am Soc Nephrol* 2009; 4: 337-344

48. Stangou M, Alexopoulos E, Papagianni A *et al*. Urinary levels of epidermal growth factor, interleukin-6 and monocyte chemoattractant protein-1 may act as predictor markers of renal function outcome in immunoglobulin A nephropathy. *Nephrology (Carlton)* 2009; 14: 613-620

49. Fornoni A, Ijaz A, Tejada T, Lenz O. Role of inflammation in diabetic nephropathy. *Curr Diabetes Rev* 2008; 4: 10-17

50. Tam FW, Riser BL, Meeran K, Rambow J, Pusey CD, Frankel AH. Urinary monocyte chemoattractant protein-1 (MCP-1) and connective tissue growth factor (CCN2) as prognostic markers for progression of diabetic nephropathy. *Cytokine* 2009; 47: 37-42

51. Brinkman JW, de Zeeuw D, Duker JJ *et al*. Falsely low urinary albumin concentrations after prolonged frozen storage of urine samples. *Clin Chem* 2005; 51: 2181-2183

52. Brinkman JW, de Zeeuw D, Lambers Heerspink HJ *et al*. Apparent loss of urinary albumin during long-term frozen storage: HPLC vs immunonephelometry. *Clin Chem* 2007; 53: 1520-1526

53. Brinkman JW, de Zeeuw D, Gansevoort RT *et al*. Prolonged frozen storage of urine reduces the value of albuminuria for mortality prediction. *Clin Chem* 2007; 53: 153-154

54. Brantsma AH, Bakker SJ, de Zeeuw D, de Jong PE, Gansevoort RT. Extended prognostic value of urinary albumin excretion for cardiovascular events. *J Am Soc Nephrol* 2008; 19: 1785-1791

55. Spoelstra-de Man AM, Brouwer CB, Stehouwer CD, Smulders YM. Rapid progression of albumin excretion is an independent predictor of cardiovascular mortality in patients with type 2 diabetes and microalbuminuria. *Diabetes Care* 2001; 24: 2097-2101

Chapter 2

Glomerular and tubular damage markers are elevated in patients with diabetes mellitus

Diabetes Care, 34: 975-981, 2011

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Abstract

We investigated in a cross-sectional study the levels of serum and urinary damage markers in patients with diabetes (n=94) and non-diabetic controls (n=45) to study the association of glomerular (IgG), proximal tubular (KIM-1, NAG, NGAL, cystatin C) and distal tubular damage (H-FABP) markers with kidney disease severity as assessed by albuminuria and eGFR.

Damage markers were measured in triplicate in fresh morning urine samples and in plasma.

Of the diabetes patients, 41 were normo-, 41 micro- and 12 macro-albuminuric. Urinary NAG (9 fold), NGAL (1.5 fold) and H-FABP (3.5 fold) were significantly elevated in normoalbuminuric diabetic patients compared to non-diabetic controls. Urinary concentrations of all markers increased per albuminuria stratum, except KIM-1. All urinary damage markers except KIM1 were significantly associated with albuminuria, independent of age, sex and plasma concentrations of the corresponding biomarker (Std βs between 0.35 and 0.87; all $p \leq 0.001$). All urinary damage markers except KIM-1 were significantly associated with eGFR in univariate models (Std βs between -0.38 and -0.21; all $p < 0.04$). After adjustment for age, sex, plasma concentration of the corresponding damage marker and albuminuria, only the association of H-FABP with eGFR remained significant (Std β -0.26, p=0.037).

Glomerular and tubular markers are associated with albuminuria, independently of eGFR, suggesting that albuminuria reflects both glomerular and tubulo-interstitial damage. Only urinary H-FABP is associated with eGFR independently of albuminuria and therefore may be a promising urinary damage marker to assess diabetic kidney disease.

Introduction

Diabetic nephropathy occurs in 20-40% of patients with diabetes mellitus and is the leading cause of chronic kidney disease and end-stage renal disease in the United States and many other western countries.[1;2] The onset of elevated levels of urinary albumin excretion is an early sign of diabetic nephropathy. Various studies have shown that in subjects with diabetes micro-albuminuria predicts the occurrence of macro-albuminuria and renal function decline.[3] As a result, high albuminuria has become an established renal risk marker in these patients.[4]

Classically, albuminuria is regarded as the consequence of diabetes-induced glomerular damage. More recently, it is increasingly appreciated that the renal tubulo-interstitium plays a role in the pathogenesis of diabetic nephropathy, with prolonged exposure to a variety of metabolic and hemodynamic injuring factors associated with sustained diabetic disease as contributing factors.[5;6] Furthermore, persistent albuminuria secondary to glomerular lesions, may be directly harmful to renal tubular cells, leading to tubular inflammation and tubulo-interstitial fibrosis.[7;8]

Several tubular damage markers have recently been discovered. Increased levels of these markers are supposed to indicate proximal tubular damage in the case of KIM-1, NGAL, NAG, cystatin C, and distal tubular damage in the case of H-FABP. These tubular damage markers have been extensively investigated in the field of predicting the occurrence of acute kidney injury after various nephrotoxic insults, such as ischemia during cardiac surgery, sepsis and administration of contrast medium.[9-11] Little research has yet been done in patients with chronic kidney disease. In this study we investigated the serum and urinary levels of the aforementioned damage markers in patients with diabetes and non-diabetic controls in order to investigate the relation of these markers to the severity of kidney disease as assessed by albuminuria and eGFR. As a secondary aim we investigated

whether these damage markers are related to eGFR independent of albuminuria.

Materials and Methods

Patients

Patients with type 1 and 2 diabetes visiting a diabetes specialty clinic were recruited. Inclusion was between April 2009 and September 2009. They were stratified by the amount of albuminuria, based on a first morning urine void. We included 94 diabetic patients, of whom 41 had normo-albuminuria, 41 had micro-albuminuria and 12 had macro-albuminuria. To ensure that macro-albuminuria was the consequence of diabetic nephropathy, patients with macro-albuminuria were required to have diabetic retinopathy. Patients with cancer, infections or inflammatory conditions, renal disease other than diabetic nephropathy, use of nephrotoxic drugs, renal transplantation or pregnancy were excluded. Patients younger than 18 years of age were also excluded. As control group we included 45 non-diabetic subjects without chronic kidney disease (1:2 versus diabetic patients). Subjects were excluded from the control group if they had a fasting glucose > 7.0 mmol/L, used glucose lowering medication, had an eGFR<60 ml/min/1.73m^2 or an ACR>30 mg/g. The study protocol was approved by the local Ethics Committee.

Measurements

We instructed patients to collect their first morning urine void on the day of their clinic visit. Blood pressure was assessed with a single measurement. Urinary albumin concentration was determined by nephelometry (BNII; Dade Behring Diagnostics, Marburg, Germany). Serum and urine creatinine were measured with an enzymatic creatinine assay (Roche, Mannheim, Germany). Glucose, HbA1c and cholesterol levels were measured with standard laboratory testing.

Definitions

We defined history of cardiovascular disease as having had myocardial infarction, stroke or surgery or endovascular treatment for coronary carotid or peripheral (legs, abdominal, aorta) artery disease. Hypertension was defined as systolic blood pressure >140 mmHg, a diastolic blood pressure > 90 mmHg or use of blood-pressure-lowering medication. Normo-albuminuria was defined as urinary albumin to creatinine ratio (ACR) <3 mg/mmol, microalbuminuria as ACR 3-30 mg/mmol and macro-albuminuria as ACR >30 mg/mmol.[12] Serum creatinine values were used to calculate an estimated glomerular filtration rate (eGFR), using the abbreviated MDRD formula.

Damage markers

We measured immunoglobulin G (IgG) as glomerular damage marker. As markers of proximal tubular damage we measured Kidney Injury Molecule 1 (KIM-1), neutrophil gelatinase-associated-lipocalin (NGAL), N-acetyl-beta-D-glucosaminidase (NAG) and Cystatin C. As a marker of distal tubular damage Heart Fatty Acid Binding Protein (H-FABP) was measured. Urinary concentrations of KIM-1, NGAL, H-FABP and IgG were measured by ELISA. For KIM-1 and NGAL, antibodies were obtained from R&D systems, Minneapolis, USA. H-FABP and IgG antibodies were obtained from Hytest, Finland. We measured urinary concentration of NAG using a modified enzyme assay according to Lockwood and corrected for nonspecific conversion (HaemoScan, Groningen, the Netherlands). Cystatin C was measured by nephelometry (reagents obtained from Siemens, Marburg, Germany). We measured all samples in triplicate in fresh samples of both urine and plasma. Urinary damage marker concentrations are expressed per mmol creatinine.

Statistical analyses

Analyses were performed with SPSS version 16.0 (SPSS Inc., Chicago, IL). Characteristics were calculated per albuminuria stratum. Parametric

variables were expressed as mean ± standard deviation (SD), whereas non-parametric variables were given as median (interquartile range). We tested pvalue for trend over albuminuria strata in diabetic patients using ANOVA for normal distributed variables. For non-normal distributed variables we used a Kruskall-Wallis test. Logarithmic transformation of damage marker excretion was applied. To investigate the association between the individual damage marker and eGFR or albuminuria we performed a linear regression analysis by using eGFR or albuminuria as independent variable and the various damage markers as dependent variable. Various models were gradually built to adjust for possible confounding. First, we investigated the crude association between damage marker and eGFR or albuminuria. Second, we performed multivariate analysis, adjusting for age and sex. Third, we tested whether plasma levels of the corresponding damage marker influenced the association between damage marker and eGFR or albuminuria. Lastly, to investigate whether knowledge on urinary damage marker concentration may be of value to assess severity of diabetic kidney disease in addition to knowledge on albuminuria status, we performed multivariable regression analysis with eGFR as dependent variable and damage markers as independent variables, adjusting for albuminuria. We added the use of diuretics, use of ACEi/ARB and type of diabetes mellitus to the final multivariable models (model 3 for albuminuria and model 4 for eGFR) as sensitivity analysis. If any of these covariates showed a significant association with either albuminuria or eGFR, we tested for interaction by entering the urinary biomarker, the investigated characteristic, and their product term in the multivariable regression model. For all analyses, a two-sided $p < 0.05$ was considered to indicate statistical significance.

Results

A total of 94 patients with DM and 45 controls participated in this study. Characteristics of patients and controls are given in table 1. Patients in the micro- and macro-albuminuric groups were more often male and smoking,

more often had a history of cardiovascular disease, used more antihypertensive medications and had a lower eGFR when compared to normo-albuminuric diabetic subjects.

Table 1 Characteristics of non-diabetic control subjects (n=45) and diabetic patients (n=94) according to albuminuria stratum

		Subjects with DM			
	Non-diabetic	*Normo*	*Micro*	*Macro*	*p-value in DM*
N	45	41	41	12	
Age (y)	53 (13)	59 (13)	64 (12)***	63 (13)*	0.12
Male gender (%)	56%	73%	66%	83%	0.48
Duration DM (y)	-	24 (11)	20 (9)	27 (8)	0.064
Type diabetes (% type 2)	-	49%	78%	83%	0.007
History CVD (%)	0%	22%	45%	64%	0.018
Smoking (%)	22%	10%	32%	33%	0.035
BMI (kg/m2)	27 (6)	30 (5)*	32 (6)**	32 (5)*	0.28
SBP (mm Hg)	132 (16)	139 (15)*	141 (17)**	152 (14)***	0.052
DBP (mm Hg)	74 (9)	78 (10)	77 (77)	77 (13)	0.90
Antihypertensive medication (%)	18%	72%***	95%***	100%***	0.006
- ACEi/ARB (%)	4%	65%***	80%***	83%***	0.24
- Diuretics	11%	32%***	63%***	75%***	0.015
Hypertension (%)	36%	85%***	97%***	100%***	0.071
HbA1c (%)	5.4 (0.3)	7.7 (1.0)***	7.6 (1.3)***	7.8 (0.7)***	0.87
eGFR MDRD (ml/min/ 1.73 m^2)	86 (14)	85 (21)	72 (22)**	55 (24)**	<0.001
ACR (mg/mmol)	0.56 (0.44-1.0)	0.70 (0.36-1.21)	8.7 (5.6-13.7)***	115 (71-130)***	<0.001

Parametric variables are expressed as mean ± standard deviation (SD) and non-parametric variables are given as median (interquartile range). *p<0.05, **p<0.01 and ***p<0.001 versus non-diabetic controls, calculated using independent sample t-test for normal distributed variables and Mann-Whitney U test for non-normal distributed variables. Abbreviations are: ACEi/ARB, ACE inhibitors or angiotensin receptor blockers; DM, diabetes mellitus; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; CVD, cardiovascular disease; ACEi/ARB, use of Angiotensin Converting Enzyme Inhibitors; ARB, Angiotensin Receptor Blockers; eGFR, estimated Glomerular Filtration Rate; ACR, Albumin Creatinine Ratio.

Damage marker concentrations

Damage marker concentrations in non-diabetic controls and in diabetic patients are shown in table 2 and figure 1. Urinary damage marker concentrations of NAG, NGAL and H-FABP were higher in normo-albuminuric patients with diabetes than in controls and increased with increasing categories of albuminuria in patients with diabetes. In contrast, cystatin C was lower in normo-albuminuric diabetic patients than in non-diabetic controls, but again increased with increasing categories of albuminuria in patients with diabetes. KIM-1 was higher in normo-albuminuric patients with diabetes than in controls, but was not different between the categories of albuminuria in patients with diabetes (Figure 1 and table 2). Differences between categories of albuminuria were most pronounced for the glomerular marker IgG (>30 fold increase from normo-albuminuria to macro-albuminuria) and the distal tubular marker H-FABP (>21 fold). Differences were less pronounced for the proximal tubular markers (from no increase to 8.5 fold for NGAL). Plasma concentrations of the various markers are given in supplemental table 1.

Table 2 Damage marker concentrations in nondiabetic control subjects and in diabetic patients according to albuminuria stratum

		Subjects with DM			
	Non-diabetic	*Normo*	*Micro*	*Macro*	*p-value in DM*
- Albumin (mg/mmol)	0.56 (0.44-1.0)	0.70 (0.36-1.21)	8.7 (5.6-13.7)***	115 (71-130)***	<0.001
Glomerular					
- IgG (µg/mmol)	465 (279-867)	242 (151-751)	1320 (590-3780)**	7379 (5079-9202)***	<0.001
Proximal tubular					
- KIM-1 (ng/mmol)	65 (33-104)	168 (116-216)***	122 (73-221)***	305 (112-417)***	0.53
- NAG (U/mmol)	0.10 (0.07-0.14)	0.9 (0.6-1.4)***	1.2 (0.9-2.1)***	2.5 (1.4-3.4)***	<0.001
- NGAL (µg/mmol)	1.3 (0.8-2.0)	2.1 (1.1-7.2)**	5.5 (2.9-14.0)***	18.0 (6.9-45.1)***	0.001
- CysC (µg/mmol)	4.2 (3.3-5.1)	2.6 (1.8-4.4)**	3.9 (2.7-5.0)	10.3 (6.7-33.3)***	<0.001
Distal tubular					
- H-FABP (ng/mmol)	34 (24-44)	130 (59-413)***	300 (104-1215)***	2742 (712-7199)***	<0.001

Data are expressed per mmol urinary creatinine concentration and given as medians (25^{th}-75^{th} percentile) *p<0.05, **p<0.01 and ***p<0.001 versus non-diabetic controls, calculated using Mann-Whitney U test.

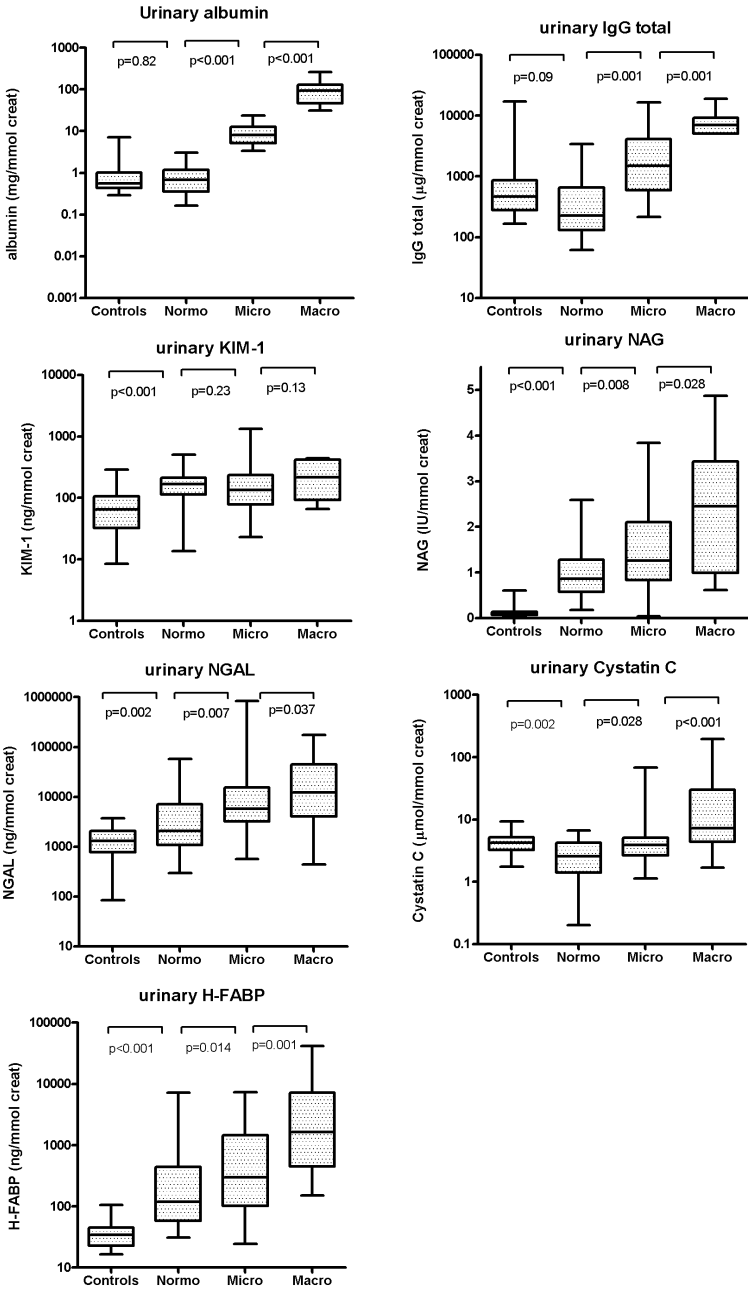

Figure 1 Biomarker concentrations in nondiabetic controls subjects and in diabetic patients, according to albuminuria stratum (normoalbuminuria, microalbuminuria and macroalbuminuria). Box plots show medians (25^{th}-75^{th} percentile). Significance was tested using the Mann-Whitney U test.

Associations of damage markers with albuminuria in diabetic patients

Associations between the various urinary damage markers and albuminuria were analyzed in all diabetic patients. All damage markers were found to be significantly associated with albuminuria in crude models, except for KIM-1 (table 3, model 1). Also, the results did not materially change when adjusted for age and sex (model 2), and additionally for the plasma concentration of the corresponding damage marker (model 3) and for eGFR (model 4). All investigated damage markers except KIM-1 remained associated with albuminuria. When information on use of diuretics, use of ACE-inhibitors/ ARB's or type of diabetes was added to the multivariable regression analyses, only the type of diabetes was a significant covariate for the association between urinary KIM-1, NAG, NGAL, cystatin C and H-FABP with albuminuria. Sex was a significant covariate in only the association between NGAL and albuminuria. We subsequently tested for interaction between these covariates and these damage markers in their association with albuminuria. Of all tested interactions terms only the one between sex and NGAL reached statistical significance.

Associations of damage markers with eGFR in diabetic patients

Table 3 also shows the associations between the various urinary damage markers and eGFR. Again, all damage markers, except KIM-1, were significantly associated with eGFR in crude models (model 1). However, when adjusted for age and sex, only albumin, the glomerular marker IgG, the proximal tubular markers NGAL and cystatin C and the distal tubular marker H-FABP remained significantly associated with eGFR (model 2). When additionally adjusted for plasma concentration of the corresponding damage marker, IgG, albumin, NGAL and H-FABP remained significant, whereas cystatin C lost significance. The association between the distal tubular marker H-FABP and eGFR was the only one that remained significant, after further adjustment for albuminuria (std β -0.26, p-value=0.037). When information on sex, use of diuretics, use of ACE-inhibitors/ARBs or type of diabetes was added to the multivariable regression analyses, only sex was a

significant covariate for the association between urinary cystatin C with eGFR, but no significant interaction was found between sex and urinary cystatin C.

Table 3 Multivariable regression analysis of various damage markers vs albuminuria or eGFR

Damage markers vs. albuminuria

	IgG		*KIM-1*		*NGAL*		*Cys C*		*NAG*		*H-FABP*	
Model	Std. β	p	Std. β	p	Std. β	p	Std. β	p	Std. β	p	Std. β	p
1	**0.78**	**<0.001**	0.15	0.195	**0.34**	**0.001**	**0.49**	**<0.001**	**0.37**	**<0.001**	**0.48**	**<0.001**
2	**0.80**	**<0.001**	0.13	0.252	**0.42**	**<0.001**	**0.51**	**<0.001**	**0.37**	**<0.001**	**0.49**	**<0.001**
3	**0.87**	**<0.001**	0.12	0.311	**0.45**	**<0.001**	**0.35**	**0.001**	**0.60**	**<0.001**	**0.50**	**<0.001**

Damage markers vs. eGFR

	Alb		*IgG*		*KIM-1*		*NGAL*		*Cys C*		*NAG*		*H-FABP*	
Model	Std. β	p	Std. β	p	Std. β	p	Std. β	p	Std. β	p	Std. β	p	Std. β	p
1	**-0.37**	**<0.001**	**-0.35**	**0.034**	-0.13	0.24	**-0.38**	**<0.001**	**-0.26**	**0.012**	**-0.32**	**0.002**	**-0.34**	**0.001**
2	**-0.32**	**<0.001**	**-0.34**	**0.026**	0.03	0.74	**-0.29**	**0.004**	**-0.28**	**0.002**	-0.19	0.053	**-0.35**	**<0.001**
3	**-0.32**	**0.001**	**-0.39**	**0.018**	-0.08	0.42	**-0.26**	**0.014**	-0.08	0.21	-0.23	0.074	**-0.36**	**0.001**
4	**-**	**-**	0.03	0.91	0.11	0.27	-0.11	0.311	0.07	0.30	0.02	0.91	**-0.26**	**0.037**

Bold print shows associations between damage markers and albuminuria or eGFR that reach statistical significance. Model : crude; model 2: adjustment for age and sex; model 3: adjustment for age, sex, and plasma concentration of the corresponding damage marker; model 4: adjustment for age, sex, plasma concentration of the corresponding damage marker and albuminuria.

Discussion

In the present study, we measured markers of glomerular, proximal tubular and distal tubular damage to study the relationship between these damage markers with the severity of diabetic kidney disease as assessed by albuminuria and estimated glomerular filtration rate. We found that urinary concentrations of glomerular, proximal tubular and distal tubular damage markers were higher when patients had more albuminuria. Interestingly, even in normo-albuminuric diabetic patients some of these markers were already elevated compared to non-diabetic subjects. In regression analyses, all damage markers that were significantly associated with albuminuria were also significantly associated with eGFR, except cystatin C and NAG. H-

FABP was the only damage marker significantly associated with eGFR after adjustment for albuminuria.

In the diabetic patients, the urinary concentration of the investigated glomerular damage marker IgG increased over the three albuminuria groups and is strongly associated with albuminuria. In healthy subjects, only trace amounts of these large molecular weight proteins are leaked by the glomerulus. These data suggest therefore that in albuminuric diabetic patients the amount of albuminuria reflects glomerular damage at least to a certain extent. Similar observations have been described before.[13;14]

In general, all investigated tubular markers except KIM-1 increased in higher albuminuria strata. Only a limited number of studies have to date investigated the association of tubular markers with the severity of chronic kidney disease in diabetic nephropathy. Our results with respect to the proximal tubular markers are similar to these studies insofar that they also showed that the urinary level of tubular damage markers parallels the degree of urinary albumin excretion.[15-18] It can be argued that this may be due to the tubulotoxic effect of albumin and other proteins that are leaked into the tubular lumen[7]. However, such tubulotoxic effects cannot fully explain our findings, since some of the markers under investigation were already increased in the normo-albuminuric diabetic patients compared to non-diabetic controls, whereas albuminuria (and eGFR) in these normo-albuminuric diabetic patients was comparable to non-diabetic controls. A limited number of other studies have previously described elevated levels of tubular markers in normo-albuminuric diabetic subjects when compared to healthy controls[17-19]. Urinary excretion of NGAL and lysosomal enzymes as NAG and cathepsin have for instance been found to be increased. These data suggest a role of the tubulo-interstitium in the pathogenesis and progression of renal damage in patients with diabetes mellitus. This assumption is strengthened by two other observations in our study. First, we found that some of these markers were associated with diabetes related

factors in normo-albuminuric diabetic subjects, such as BMI, duration of diabetes and metabolic regulation. Second, to our knowledge we are the first to show that a marker of distal tubular damage, H-FABP, is also associated with albuminuria, despite the fact that the distal tubule is assumed less sensitive to toxic effects of urinary proteins.

Data in literature also support that in diabetic kidney disease the severity of chronic kidney disease depends not only on the severity of glomerular lesions, but also on tubulo-interstitial damage. A histological study showed that proximal tubular basement membrane width is already thickened compared to healthy controls in normo-albuminuric diabetic patients.[20] Another study in micro-albuminuric diabetic patients with similar GFR showed that only 29% had typical histological glomerular features of diabetic nephropathy, whereas 42% had severe tubulo-interstitial lesions disproportional to the mild glomerular involvement.[21]

All markers that were associated with albuminuria were also correlated with eGFR in crude models. However, the association of eGFR with urinary NAG concentration lost significance after adjustment for age and sex, and the association with urinary cystatin C lost significance after additional adjustment for its plasma concentration. In healthy subjects cystatin C is a marker for eGFR, with impaired GFR being associated with higher levels of cystatin C. Our data imply that the urinary concentration of cystatin C is largely determined by the amount that is filtrated through the glomerulus in subjects with diabetic nephropathy.

As secondary aim in our study, we investigated which damage markers are associated with eGFR independent of albuminuria. Albuminuria is widely acknowledged to be associated with eGFR in subjects with diabetic nephropathy.[3;22] We found that the associations of all markers under investigation with eGFR lost statistical significance when adjusted for albuminuria, except for HFABP. In addition, we found that urinary H-FABP concentration was already significantly elevated in normo-albuminuric patients with diabetes mellitus compared to non-diabetic control patients

whom had a similar eGFR. These data suggest that urinary H-FABP concentration is a promising marker to predict clinical outcome of diabetic nephropathy in addition to albuminuria.

We acknowledge that the present study has limitations. First, the present study is a single-centre study and the association of the measured damage markers with albuminuria and eGFR needs to be confirmed. Second, this study has a cross-sectional design. Whether or not the damage markers as assessed in our study predict progression of diabetic nephropathy has to be investigated in longterm, prospective, observational studies. Lastly, a larger number of patients would have allowed us to perform analyses in specific subgroups. However, the results of our multivariable regression analyses in which interactions were tested showed that there were almost no significant interactions between these covariates and the various urinary damage markers in their association with albuminuria or eGFR. This suggests that the associations between these damage markers and albuminuria or eGFR are not different in men versus women, in subjects using or not using diuretics, in subjects using or not using ACEi/ARBs, nor in type 1 versus type 2 diabetes mellitus. Strengths of this study are that we measured a wide range of kidney damage markers, representing different parts of the nephron, ranging from glomerulus, proximal to distal tubule. Furthermore, we measured all our markers in fresh urine samples to assure optimal sample quality. It has previously been shown for several markers that frozen storage and freeze-thaw cycles lead to a systematic decrease and increase in variability,[9;23;24] which may negatively influence association studies such as the present one. Finally, most authors investigating urinary excretion of renal damage markers did not measure plasma concentrations.[9;25] We also measured the corresponding plasma concentration of all urinary markers to rule out that a high plasma concentration instead of renal damage causes a high urinary concentration. This appeared to be the case for cystatin C.

In conclusion, urinary concentrations of most investigated damage markers are elevated in patients with diabetes mellitus when compared to non-diabetic controls. These damage markers are associated with the severity of diabetic nephropathy as assessed by albuminuria and eGFR. Interestingly, some of these markers are already elevated in normo-albuminuric diabetic patients with normal eGFR. This renders these proteins as potential sensitive markers of early diabetic kidney damage. Only the distal tubular marker H-FABP was found to be associated with eGFR independent of albuminuria, suggesting that especially measuring urinary H-FABP concentration may be useful to assess severity of diabetic kidney damage in addition to measuring albuminuria.

Statement of competing financial interests

None of the authors has anything to declare.

Acknowledgements

The authors thank Katinka Mulder (University Medical Center Groningen) for help with reviewing the manuscript.

Reference List

1. KDOQI Clinical Practice Guidelines and Clinical Practice Recommendations for Diabetes and Chronic Kidney Disease. *Am J Kidney Dis* 2007; 49: S12-154

2. Standards of medical care in diabetes--2007. *Diabetes Care* 2007; 30 Suppl 1: S4-S41

3. O'Hare AM, Hailpern SM, Pavkov ME *et al*. Prognostic implications of the urinary albumin to creatinine ratio in veterans of different ages with diabetes. *Arch Intern Med* 2010; 170: 930-936

4. Mogensen CE, Christensen CK. Predicting diabetic nephropathy in insulin-dependent patients. *N Engl J Med* 1984; 311: 89-93

5. Thomas MC, Burns WC, Cooper ME. Tubular changes in early diabetic nephropathy. *Adv Chronic Kidney Dis* 2005; 12: 177-186

6. Comper WD, Hilliard LM, Nikolic-Paterson DJ, Russo LM. Disease-dependent mechanisms of albuminuria. *Am J Physiol Renal Physiol* 2008; 295: F1589-F1600

7. Abbate M, Zoja C, Remuzzi G. How does proteinuria cause progressive renal damage? *J Am Soc Nephrol* 2006; 17: 2974-2984

8. Zoja C, Garcia PB, Remuzzi G. The role of chemokines in progressive renal disease. *Front Biosci* 2009; 14: 1815-1822

9. Han WK, Wagener G, Zhu Y, Wang S, Lee HT. Urinary biomarkers in the early detection of acute kidney injury after cardiac surgery. *Clin J Am Soc Nephrol* 2009; 4: 873-882

10. Haase M, Bellomo R, Devarajan P, Schlattmann P, Haase-Fielitz A. Accuracy of neutrophil gelatinase-associated lipocalin (NGAL) in diagnosis and prognosis in acute kidney injury: a systematic review and meta-analysis. *Am J Kidney Dis* 2009; 54: 1012-1024

11. Bagshaw SM, Bellomo R. Early diagnosis of acute kidney injury. *Curr Opin Crit Care* 2007; 13: 638-644

12. Levey AS, Eckardt KU, Tsukamoto Y *et al*. Definition and classification of chronic kidney disease: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). *Kidney Int* 2005; 67: 2089-2100

13. Satchell SC, Tooke JE. What is the mechanism of microalbuminuria in diabetes: a role for the glomerular endothelium? *Diabetologia* 2008; 51: 714-725

14. Jefferson JA, Shankland SJ, Pichler RH. Proteinuria in diabetic kidney disease: a mechanistic viewpoint. *Kidney Int* 2008; 74: 22-36

15. Bolignano D, Coppolino G, Campo S *et al*. Urinary neutrophil gelatinase-associated lipocalin (NGAL) is associated with severity of renal disease in proteinuric patients. *Nephrol Dial Transplant* 2008; 23: 414-416

16. Piwowar A, Knapik-Kordecka M, Fus I, Warwas M. Urinary activities of cathepsin B, N-acetyl-beta-D-glucosaminidase, and albuminuria in patients with type 2 diabetes mellitus. *Med Sci Monit* 2006; 12: CR210-CR214

17. Mohammadi-Karakani A, Asgharzadeh-Haghighi S, Ghazi-Khansari M, Hosseini R. Determination of urinary enzymes as a marker of early renal damage in diabetic patients. *J Clin Lab Anal* 2007; 21: 413-417

18. Bolignano D, Lacquaniti A, Coppolino G *et al*. Neutrophil gelatinase-associated lipocalin as an early biomarker of nephropathy in diabetic patients. *Kidney Blood Press Res* 2009; 32: 91-98

19. Uslu S, Efe B, Alatas O *et al*. Serum cystatin C and urinary enzymes as screening markers of renal dysfunction in diabetic patients. *J Nephrol* 2005; 18: 559-567

20. Brito PL, Fioretto P, Drummond K *et al*. Proximal tubular basement membrane width in insulin-dependent diabetes mellitus. *Kidney Int* 1998; 53: 754-761

21. Fioretto P, Mauer M, Brocco E *et al*. Patterns of renal injury in NIDDM patients with microalbuminuria. *Diabetologia* 1996; 39: 1569-1576

22. Keane WF, Zhang Z, Lyle PA *et al*. Risk scores for predicting outcomes in patients with type 2 diabetes and nephropathy: the RENAAL study. *Clin J Am Soc Nephrol* 2006; 1: 761-767

23. Lambers Heerspink HJ, Nauta FL, van der Zee CP *et al*. Alkalinization of urine samples preserves albumin concentrations during prolonged frozen storage in patients with diabetes mellitus. *Diabet Med* 2009; 26: 556-559

24. Haase-Fielitz A, Haase M, Bellomo R. Instability of urinary NGAL during long-term storage. *Am J Kidney Dis* 2009; 53: 564-565

25. Hofstra JM, Deegens JK, Willems HL, Wetzels JF. Beta-2-microglobulin is superior to N-acetyl-beta-glucosaminidase in predicting prognosis in idiopathic membranous nephropathy. *Nephrol Dial Transplant* 2008;

Supplemental table

Table 1 Plasma damage marker concentrations of patients with diabetes mellitus according to albuminuria stratum

	Normo-albuminuria	*Micro-albuminuria*	*Macro-albuminuria*	*p-value for trend in DM*
Glomerular				
- IgG (mg/ml)	6.3 (4.4-40.7)	21.8 (5.2-39.9)	7.0 (2.2-32.2)	0.80
Proximal tubular				
- KIM-1 (ng/ml))	0.05 (0.0-0.32)	0.16 (0.07-0.30)	0.19 (0.07-0.28)	0.88
- NAG (U/L)	31.8 (23.3-39.7)	32.6 (24.5-48.2)	23.2 (18.5-35.3)	0.19
- NGAL (ng/ml)	197 (117-323)	188 (131-288)	245 (193-430)	0.52
- CysC (mg/L)	0.79 (0.68-0.89)	1.00 (0.80-1.17)	1.30 (1.17-1.44)	<0.001
Distal tubular				
- H-FABP (ng/ml)	0.84 (0.36-2.63)	1.11 (0.00-2.53)	1.94 (0.47-17.08)	0.001

Values are given as median (25^{th}-75^{th} percentile)

Chapter 3

Alkalinization of urine samples preserves albumin concentrations during prolonged frozen storage in patients with diabetes mellitus

Diabetic Medicine, 26: 556-559, 2009

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Abstract

In epidemiological studies in patients with diabetes, urine samples are often stored frozen prior to assessment of urinary albumin concentration (UAC). However, prolonged frozen storage may result in a falsely low urinary albumin concentration. In the current study, we investigated whether adjustment of urinary pH to alkaline values prior to frozen storage can prevent this problem. Urine samples were collected in 90 patients from our diabetes outpatient clinic and divided into two portions. One portion was first adjusted to pH > 8.0 with 0.1M sodiumhydroxide, the other was left unprocessed. Both portions were divided into aliquots. Urinary albumin concentration was assessed in fresh samples and after 7 days, 1, 6 and 12 months of storage at -20 and -80 ºC.

Until 1 month of storage there were no significant changes in urinary albumin concentration. After longer storage, urinary albumin concentration fell significantly in pH unadjusted samples stored at -20 ºC, with a -7.6% (27.8) and -13.6% (31.7) change after 6 and 12 months storage, respectively. No significant change in UAC occurred in pH adjusted samples stored at -20 ºC or when samples were stored at -80 ºC, both with and without pH adjustment. Variation in UAC assessed after 12 months of storage was larger for samples stored at -20 ºC without adjustment of pH than for the samples stored with pH adjustment or stored at -80 ºC.

Urine alkalinization to pH > 8.0 prevents the decline in UAC associated with 12 months of frozen storage at -20 ºC and results in lower variation between samples after storage.

Introduction

In today's practice of diabetes care, albuminuria has become an important marker for assessment of cardiovascular and renal risk and, as such, is used as a predictor of events and an intermediate endpoint in epidemiological studies and intervention studies respectively.[1-3] In epidemiological and intervention studies, urine samples are often stored frozen at -20º C for prolonged periods of time before assessment of urinary albumin concentration (UAC).[4;5] The advantages are increased control on logistics and prevention of analytical day-to-day variation and drift. Although some studies found no effect of frozen storage on UAC[6-8], others found erroneously low values, in particular if storage was for 6 months or longer.[9-12] Importantly, the decline in UAC during frozen storage has been reported to vary greatly between samples, making the decline for an individual sample very unpredictable.[13;14] This variation in decline ultimately impairs the prognostic property of increased urinary albumin levels and results in loss of power for detection of effects on albuminuria as an intermediate endpoint in intervention studies.[15] In a previous study, we found that urinary pH is a determinant of the decline in UAC during frozen storage at -20º C, with the largest declines at pH 5.0, whereas albumin in urine with pH 8.0 did not decline.[16] In the current study, we investigated whether adjustment of urinary pH to > 8.0 prior to freezing prevents the decline in UAC during frozen storage at -20º C. Other aims were to investigate whether urinary alkalinization reduces the variation in decline in UAC and to make a comparison with storage at -80º C.

Patients and methods

Study population and urine samples

Urine samples were collected from patients with diabetes mellitus (both type 1 and type 2), who visited the outpatient diabetes clinic at the University Medical Center Groningen. During their visit to the outpatient department, participants were instructed to collect a spot midstream urine sample. Samples with a fresh urinary albumin concentration <5.0 mg/l were excluded

to ensure that a potential decline in urinary albumin concentration could be detected, given the lower limit of detection of the assay of 2.4 mg/l. Urine samples were divided into two portions. One portion was adjusted to pH > 8.0 with 1 m sodium hydroxide; the other portion was left unprocessed. The amount of added sodium hydroxide was calculated by weighing the urine sample before and after addition of sodium hydroxide. This was carried out to correct UAC for the added volume. Both urine portions were subsequently divided into several aliquots. Urinary albumin was assessed in fresh, pH unadjusted and adjusted urine samples within 2 h after collection (reference value). Other portions of fresh urine were stored in polystyrene vials for assessment after storage at -20 and -80º C for 7 days, 1, 6 and 12 months. In addition, the intra-assay coefficient of variation (CV) was calculated in fresh pH adjusted and unadjusted samples. To reassess the intra-assay CV after 12 months, three portions of both pH adjusted and pH unadjusted urine samples were stored at -20 and -80º C. Handling of fresh and frozen urine samples, including hand inversion and centrifuging before analysis by nephelometry, was performed as described previously.[16]

Laboratory methods Urinary albumin concentration was measured using a Behring BN™ II immunonephelometer (Dade Behring, Marburg, Germany). The variability encountered with calibration and reagent lots ranged between 1.6–4.5 and 1.1–2.4%, respectively. The intra- and interassay CV were 2.1% and 4.5% respectively.[17] The lower limit of detection was 2.4 mg/l. To assess the change in albumin : creatinine ratio over time, urinary creatinine concentrations were also measured. Urinary creatinine was determined by an enzymatic method (Modular Analytics SWA; Roche, Tokyo, Japan) with intra-assay and interassay CV evaluated in our laboratory of 1.9% and 2.1%, respectively. Urinary pH was determined by a combined pH electrode connected to a C833 multi-channel analyser (Consort, Turnhout, Belgium).

Statistical analyses

Data are presented as mean ± standard deviation, or mean (range) where appropriate. In case of skewed distribution, data are presented as median

(interquartile range). Differences among groups were assessed by anova and differences between groups by post-hoc analysis according Tukey. Linear regression analysis was used to assess the association between baseline urinary pH and percentage change in urinary albumin concentration after 12 months' frozen storage. The amount of variation in assessed UAC after frozen storage was calculated for each storage condition with the formula: P absolute (percentage change in UAC individual sample—mean change UAC) and tested for statistical significance by means of a paired Student's t-test. Two sided P-values ≤ 0.05 were considered to indicate statistical significance.

Results

Ninety urine samples from patients with diabetes [age 50.4 ± 16.8 years, 65.1% males, glycated haemoglobin (HbA1c) 8.3 ± 1.3%, systolic blood pressure 138 ± 17 mmHg] were collected between July and December 2006. Fresh unadjusted urinary pH was 5.9 (range 4.8–7.4). To alkalinize the urine samples, 0.02 ± 0.016 ml 1 m sodium hydroxide was added per ml urine, resulting in a urinary pH of 8.4 (range 8.0–9.7) after adjustment ($P < 0.001$ vs. fresh). Before adjustment of pH, fresh UAC and urinary creatinine concentration were 15.5 mg/l (8.6–39.4) and 1.25 ± 0.7 g/l, respectively, compared with 15.6 mg/l (8.5–38.9) and 1.22 ± 0.7 g/l after adjustment ($P = 0.67$ for difference in UAC and $P = 0.10$ for difference in urinary creatinine concentration). Percentage changes in UAC during frozen storage are shown in Fig. 1. Until 1 month of storage, no significant changes were observed compared with fresh samples (reference values). In samples that were stored at -20º C without adjustment of pH, UAC decreased significantly by 7.6 ± 27.8 and 13.6 ± 31.7% after 6 and 12 months of storage, respectively.

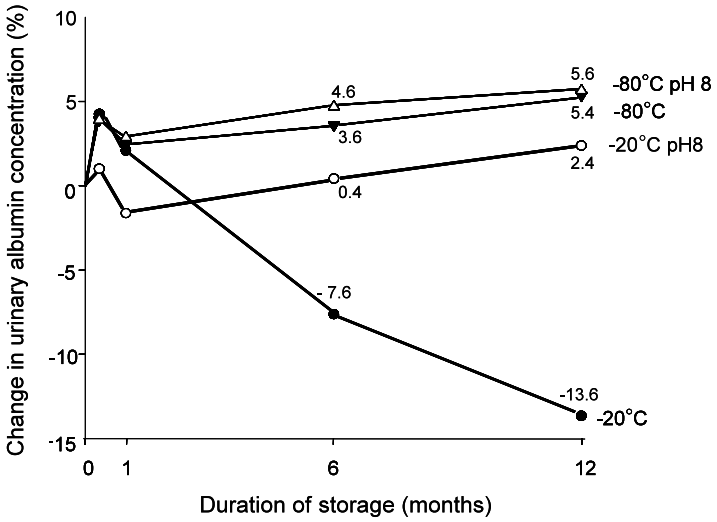

Figure 1 Percentage change in urinary albumin concentration (UAC) during the study in pH unadjusted and in adjusted samples stored at -20 and -80 ºC. Percentage change in UAC compared with fresh samples after 6 and 12 months' frozen storage are shown.

Baseline urinary pH significantly predicted change in UAC in these samples, both at 6 months (R^2 = 0.10, P < 0.01) and 12 months (R^2 = 0.18, P < 0.01) of storage. In samples in which pH was adjusted prior to storage at -20º C, decline in UAC was completely prevented, both at 6 and 12 months of storage (Fig. 1). The same was true for samples stored at -80º C, irrespective whether pH was adjusted or not. The percentage change in UAC assessed after 6 and 12 months' frozen storage varied widely in samples stored at -20º C without prior pH adjustment (Fig. 2a; data for 12 months storage are shown). The variation in change in UAC after 6 and 12 months' storage at -20º C was, to a large extent, prevented in urine samples in which the pH was adjusted. The Σ absolute (percentage change in UAC individual sample—mean change UAC) after 12 months of storage at -20º C in pH unadjusted and adjusted samples was 2315% and 715%, respectively (P < 0.001). Storage at -80º C also reduced the variation in change in UAC after 12 months, irrespective of whether pH was adjusted or not (both P < 0.001 vs. storage at -20º C).The intra-assay CV after 12 months of storage

at -20º C was slightly higher compared with fresh samples (4.2% vs. 2.1%, respectively; P < 0.01), whereas it remained unchanged in alkalinized samples stored at -20º C (2.9% vs. 2.7%, respectively; P = 0.61) or samples stored at -80º C (2.6% vs. 2.1%, P = 0.27).

Current diabetes and nephrology guidelines advocate measurement of the urinary albumin : creatinine ratio rather than UAC alone.[1;18] To determine change in the albumin : creatinine ratio, we measured urinary creatinine concentrations. Change in urinary creatinine concentration after 12 months at the various storage conditions was -0.8 ± 10.9% at -20º C, 0.4 ± 4.5% at -20º C pH > 8.0, 1.2 ± 4.8% at -80º C and 2.6 ± 8.9% at -80º C pH> 8.0 (P = NS vs. zero for all storage conditions). As urinary creatinine concentrations did not change significantly, the percentage change in the albumin : creatinine ratio after 12 months' storage was comparable with the percentage change in UAC (Fig. 2b).

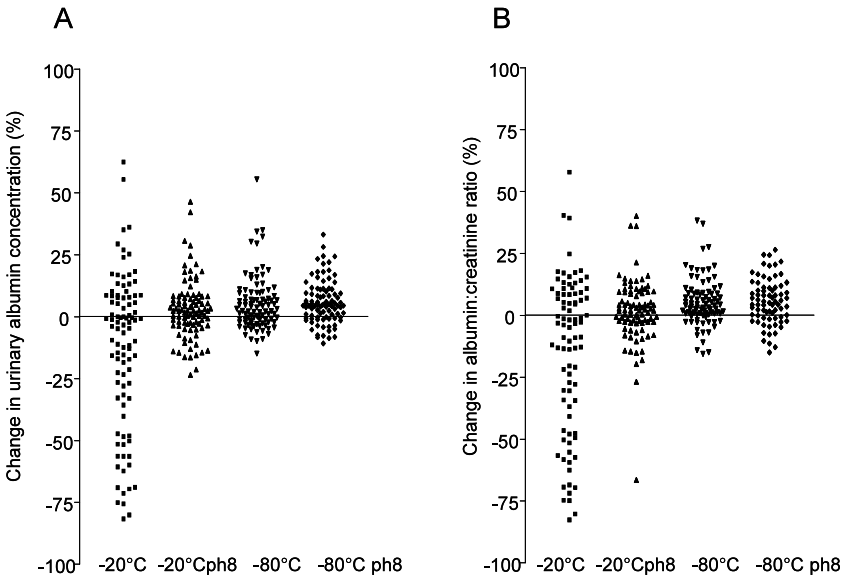

Figure 2 Percentage change in UAC and albumin:creatinine ratio after 12 months storage in pH unadjusted and adjusted samples stored at -20°C and -80°C.

Discussion

In the present study, we found that urine alkalinization to pH > 8.0 completely prevents the decline in UAC otherwise observed during prolonged frozen storage of urine samples of patients with diabetes at -20º C. Importantly, after alkalinization, storage at -20º C was equivalent to storage at -80º C, both in terms of recovery of urinary albumin and variability between individual samples. Urine alkalinization is a relatively simple and cheap method to preserve urine for prolonged frozen storage. In this study, a mean of 0.02 ml 1 mNaOH, equivalent to three droplets 1 m NaOH, was added per ml of urine. From a practical point of view, we recommend the addition of three droplets 1 m NaOH per ml urine to alkalinize samples instead of precise titration to pH 8.0. Interestingly, the intra-assay CV for samples stored at -20º C was almost similar compared with fresh samples. These data thus imply that the decline in UAC is similar in each portion of the same urine sample and is not caused by random variability. Adequate mixing of samples after thawing allows storage of urine for up to 3 months at -20º C without large changes in UAC, whereas inadequate mixing results in significant declines.[11] Some studies report a significant decline in UAC after storage durations of only up to 8 weeks.[10;19;20] As it is not mentioned in these studies whether samples were mixed, the decline in UAC reported in these studies could be the consequence of inadequate mixing of samples prior to analysis. One of these studies reported no decline in UAC in pH adjusted samples, although no actual data on UAC before and after storage were provided.[20] Furthermore, the limited sample size of that study (n = 33) and short duration of storage hampers interpretation and applicability for future studies, as samples in epidemiological and intervention studies are usually meant to be stored for longer periods of time. The study also lacked a comparison with -80º C, thus leaving open the question whether storage at -80º C was superior to storage at -20º C with adjustment of pH.

What could be the mechanism underlying prevention of decline in UAC during frozen storage by urine alkalinization? First, because the iso-electric

point of albumin is 4.7, relatively less aggregationmay occur at a higher pH. This probably results in less entrapment of albumin in the precipitate andmore efficient antibody binding, which is needed for determination by immunochemical methods. Second, urine alkalinization may prevent conformational changes of albumin resulting from frozen storage and as such improve antibody binding required for immunochemical determination. Third, it is also possible that falsely low urinary albumin concentration values reflect contamination with bacterial proteases.[17] Bacterial proteases may hydrolyse urinary albumin. Alkalinization of urine before storage may reduce activity of bacterial proteases, leading to improved preservation for albumin assessment. Unfortunately, bacterial protease activity was not measured in our study and further studies are warranted to investigate this possibility.

In conclusion, urinary alkalinization to pH > 8.0 is a simple technique to prevent a decline in UAC during frozen storage at -20º C and a suitable alternative for storage at -80º C, both in terms of recovery of urinary albumin and in variation between individual samples. The latter is particularly important for maximization of power for detection of effects of treatment in intervention studies in which urinary albumin is used as an intermediate endpoint.

Statement of competing financial interests

None of the authors has anything to declare.

Acknowledgement

We thank Jacko J. Duker (laboratory technician) for his concise and elaborate work. We thank Dade Behring (Marlburg, Germany) for supplying equipment (Behring Nephelometer II) and reagents for nephelometric measurement of urinary albumin concentration.

Reference List

1. Standards of medical care in diabetes--2008. *Diabetes Care* 2008; 31 Suppl 1: S12-S54

2. Viberti GC, Hill RD, Jarrett RJ, Argyropoulos A, Mahmud U, Keen H. Microalbuminuria as a predictor of clinical nephropathy in insulin-dependent diabetes mellitus. *Lancet* 1982; 1: 1430-1432

3. Mogensen CE. Microalbuminuria predicts clinical proteinuria and early mortality in maturity-onset diabetes. *N Engl J Med* 1984; 310: 356-360

4. Roest M, Banga JD, Janssen WM *et al*. Excessive urinary albumin levels are associated with future cardiovascular mortality in postmenopausal women. *Circulation* 2001; 103: 3057-3061

5. Yuyun MF, Khaw KT, Luben R *et al*. A prospective study of microalbuminuria and incident coronary heart disease and its prognostic significance in a British population: the EPIC-Norfolk study. *Am J Epidemiol* 2004; 159: 284-293

6. Innanen VT, Groom BM, de Campos FM. Microalbumin and freezing. *Clin Chem* 1997; 43: 1093-1094

7. Collins AC, Sethi M, MacDonald FA, Brown D, Viberti GC. Storage temperature and differing methods of sample preparation in the measurement of urinary albumin. *Diabetologia* 1993; 36: 993-997

8. Giampietro O, Penno G, Clerico A, Cruschelli L, Cecere M. How and how long to store urine samples before albumin radioimmunoassay: a practical response. *Clin Chem* 1993; 39: 533-536

9. Elving LD, Bakkeren JA, Jansen MJ, Kat Angelino CM, de Nobel E, van Munster PJ. Screening for microalbuminuria in patients with diabetes mellitus: frozen storage of urine samples decreases their albumin content. *Clin Chem* 1989; 35: 308-310

10. Osberg I, Chase HP, Garg SK *et al*. Effects of storage time and temperature on measurement of small concentrations of albumin in urine. *Clin Chem* 1990; 36: 1428-1430

11. Brinkman JW, de Zeeuw D, Duker JJ *et al*. Falsely low urinary albumin concentrations after prolonged frozen storage of urine samples. *Clin Chem* 2005; 51: 2181-2183

12. Parekh RS, Kao WH, Meoni LA *et al*. Reliability of urinary albumin, total protein, and creatinine assays after prolonged storage: the Family Investigation of Nephropathy and Diabetes. *Clin J Am Soc Nephrol* 2007; 2: 1156-1162

13. Lambers Heerspink HJ, Brinkman JW, Bakker SJ, Gansevoort RT, de Zeeuw D. Update on microalbuminuria as a biomarker in renal and cardiovascular disease. *Curr Opin Nephrol Hypertens* 2006; 15: 631-636

14. Brinkman JW, de Zeeuw D, Lambers Heerspink HJ *et al*. Apparent loss of urinary albumin during long-term frozen storage: HPLC vs immunonephelometry. *Clin Chem* 2007; 53: 1520-1526

15. Brinkman JW, de Zeeuw D, Gansevoort RT *et al*. Prolonged frozen storage of urine reduces the value of albuminuria for mortality prediction. *Clin Chem* 2007; 53: 153-154

16. Brinkman JW, Heerspink HL, de Zeeuw D, Gansevoort RT, Bakker SJ. Urinary pH affects albumin concentrations after prolonged frozen storage. *Nephrol Dial Transplant* 2007; 22: 3670

17. Rowe DJ, Dawnay A, Watts GF. Microalbuminuria in diabetes mellitus: review and recommendations for the measurement of albumin in urine. *Ann Clin Biochem* 1990; 27 (Pt 4): 297-312

18. KDOQI Clinical Practice Guidelines and Clinical Practice Recommendations for Diabetes and Chronic Kidney Disease. *Am J Kidney Dis* 2007; 49: S12-154

19. d'Eril GM, Valenti G, Pastore R, Pankopf S. More on stability of albumin, N-acetylglucosaminidase, and creatinine in urine samples. *Clin Chem* 1994; 40: 339-340

20. Townsend JC, Blair PJ, Forrest AR. Effect of storage pH on precipitation of albumin from urine from diabetics. *Clin Chem* 1988; 34: 1355-1356

Chapter 4

The effect of frozen storage on urinary renal damage markers concentration

Accepted for publication in revised form in

American Journal of Kidney Disease

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Abstract

Little is known about the effect of frozen storage on urinary concentrations of renal damage markers. We therefore investigated the effect of storage at -20 and -80 °C on concentration and variability of these markers.

Urine samples of 95 patients with diabetes mellitus were collected, in which we measured IgG, IgG-4, KIM-1, NGAL, NAG, cystatin C and H-FABP. Damage marker concentrations were first measured in fresh samples. Subsequently urine samples were frozen at -80 ºC and remeasured after 1 week, 6 months and 1 year. Furthermore, the effect of 1 year frozen storage at -20 ^{0}C and the effect of specific storage protocols (adding protease inhibitors, and alkalizing before and after storage) were investigated.

Average marker concentrations showed a decrease after frozen storage at -80 ºC, with KIM-1, NAG and NGAL concentrations being significantly lower after 1 year. Also, an increase in variability was observed. Specific storage protocols could not prevent this. Importantly, after frozen storage all marker concentrations remained significantly associated with the fresh baseline concentrations (all $p<0.001$).

Renal damage markers should preferably be measured in fresh urine samples. If this is not possible, urine samples can be stored at -80º C or -20º C. Frozen storage is, however, associated with a reduction in average value and an increase in variability of most markers. Marker studies using frozen urine samples should therefore be interpreted with caution.

Introduction

Measurement of urinary concentrations of tubular damage markers has achieved growing attention to predict the outcome of acute kidney damage.[1] More recently the value of these markers to predict the course of chronic renal disease has also been investigated.[2-4] Urine samples that are used for these studies are often stored frozen during prolonged periods of time prior to analysis.

Previously we have shown that frozen storage during 1 year is associated with a decrease in urinary albumin concentration, with a wide variation between urine samples in the amount to which albumin decreases.[5;6] Furthermore we have shown that this effect is more outspoken after storage at -20 ^{0}C when compared to storage at -80 ^{0}C[6,7], and that alkalinisation of urine samples before freezing at -20 ºC prevents to a certain extent the average decrease in albumin concentration and the increase in variability.[7] These storage effects are of importance, as we showed that the value of urinary albumin excretion to predict mortality is considerably better when assessed in fresh urine samples in comparison to when assessed in the same samples after prolonged frozen storage.[8] As yet little is known about the effect of frozen storage on concentration and variability of other urinary renal damage markers.

With this study we aimed to investigate the effect of frozen storage at -20 and -80 °C on concentration and variability of various urinary renal damage markers. For this study we investigated the glomerular markers IgG and IgG-4, the proximal tubular markers KIM-1, NAG, NGAL, cystatin C and the distal tubular marker H-FABP. Secondly we investigated whether specific storage protocols influence the concentration and variability in these markers after one year frozen storage.

Materials and Methods

Study population and samples

This study was performed in patients with diabetes mellitus (both Type 1 and Type 2), who consecutively visited a diabetes specialty clinic. Inclusion was between April 2009 and September 2009, and was stratified by the amount of albuminuria measured in a fresh first morning void urine sample by nephelometry (BNII; Dade Behring Diagnostics, Marburg, Germany). We aimed to include 100 diabetic patients, of which 40 with normo-albuminuria (<3mg/mmol), 40 with micro-albuminuria (3-30mg/mmol) and 20 with macro-albuminuria (> 30mg/mmol).[4] This stratification was done in order to obtain a wide variation in urinary renal damage markers. Patients with cancer, infections or inflammatory conditions, renal disease other than diabetic nephropathy, use of nephrotoxic drugs, renal transplantation or pregnancy were excluded, as were patients younger than 18 years of age. This study protocol was approved by the institutional Ethics Committee.

Study design and sample handling

Patients were invited to participate at regular clinic visits. After having obtained written informed consent patients were instructed to collect a first morning urine void at the day of visit to a research clinic, and to bring this sample to the clinic. During the visit demographic and anthropometric data were obtained. Urine samples were vortexed and subsequently centrifuged (14.000 rpm, 20.817 g). The supernatant was used for further studies. Concentrations of the various renal damage markers were measured the same day as the urine collection. Furthermore, urine samples were divided and stored in polystyrene vials for assessment after 1 year frozen storage at -20 ºC and -80 ºC. Samples that were stored at -80 ºC were also assessed after 1 week and 6 months of storage to investigate the time course of possible changes in urinary concentration and variability of the markers under study. In addition, the effect of three specific storage protocols were investigated in samples stored during 1 year at -80º C: a. alkalinisation before frozen storage by adjusting urinary pH to > 8.0 with 1 M sodium

hydroxide (primary alkalinisation), b. alkalinisation after thawing, similarly by adjusting urinary pH to > 8.0 with 1 M sodium hydroxide (secondary alkalinisation), and c. adding a cocktail of protease-inhibitors before frozen storage. Protease inhibitors that were added were leupeptin (1 µmol/l), NaN3 (10 mmol/l) and phenylmethanesulfonyl fluoride PMSP (0.5mM).[9]

Measurements of Damage Markers

Urinary renal damage markers were measured in fresh urine samples in triplicate to calculate the intra-assay coefficient of variation. Inter-assay coefficient of variation was determined by measuring the same sample three times using different assays. For quantification of immunoglobuline G, immunoglobuline G type 4, kidney injury molecule 1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), and heart-type fatty acid-binding protein (H-FABP) we used direct sandwich-enzyme-linked immunosorbent assays using monoclonal coating antibodies and labeled polyclonal detection antibodies on a Maxisorp plate (Nunc, Denmark) in which the concentration of the analyte was determined spectrophotometrically by conversion of *o*-phenylenediamine by Horse-Radish Peroxidase label. IgG, IgG-4 and H-FABP antibodies were obtained from Hytest (Turku, Finland) and KIM-1 and NGAL antibodies were obtained from R&D systems (Minneapolis, USA)). Urinary concentration of N-acetyl-β-D-glucosaminidase (NAG) was measured using a modified enzyme assay according to Lockwood and corrected for nonspecific conversion (HaemoScan, Groningen, The Netherlands). Cystatin C was measured by nephelometry (reagents obtained from Siemens (Marburg, Germany). The intra- and interassay coefficients of variation were for IgG 13.3% and 25.1%, respectively, for IgG-4 14.0% and 15.8%, for KIM-1 7.4% and 14.5%, for NAG 3.1% and 13.7%, for cystatin C 2.7% and 8.4%, for NGAL 6.8% and 19.6%, and for H-FABP 9.3% and 17.6%. Samples were diluted to obtain optimal concentration for measurement.

Statistical analyses

Statistical analyses were performed with SPSS version 18.0 (SPSS Inc., Chicago, IL). Parametric variables are expressed as mean ± standard deviation (SD), whereas non-parametric variables are given as median (interquartile range). P-values for differences between renal damage marker concentrations when measured in fresh samples compared to when measured in samples after frozen storage were assessed using the Wilcoxon Signed Rank test. Intra-assay coefficients of variation are calculated as SD / mean of 3 measurements performed in one assay and are expressed as a percentage. Inter-assay coefficients of variation are calculated as SD / mean of 3 separate measurements of the same sample in 3 different assays and similarly expressed as a percentage. Linear regression analysis was used to assess the correlation between concentrations of the various urinary renal damage markers when measured in fresh urine samples and when measured after different modalities of frozen storage. To fulfil the requirements for linear regression analysis all urinary damage marker concentrations were 10log-normalized. Because the R^2 value of the regression analysis is a measure of the strength of the linear associations rather than a measure of agreement, we also assessed agreement with the Bland Altman method.[10] Bland Altman agreements are calculated by dividing the difference between concentration measured from fresh samples and the concentrations measured from frozen stored samples by the mean of the fresh concentrations and concentration measured from frozen samples. Smaller values indicate better agreement. For all analyses a two sided $p < 0.05$ was considered to indicate statistical significance.

Results

In total 95 patients were included (67 males and 28 females). Their mean age was 61 ± 13 years, and mean systolic and diastolic blood pressure were 142 ±16 and 77±10 mmHg, respectively. Of these subjects 41 were normo-albuminuric, 41 micro-albuminuric and 13 macro-albuminuric.

Table 1 Concentration of various renal damage markers

	Fresh	-80 ^{0}C		
		1 week	*6 months*	*1 year*
IgG-total (µg/ml)	6.4 (2.1-28.9)	5.7 (1.9-10.7)*	5.2 (3.4-11.0)	5.0 (2.2-15.3)
IgG-4 (µg/ml)	1.2 (0.9-2.6)	0.8 (0.3-2.5)	0.6 (0.6-0.7)**	0.7 (0.3-2.9)
KIM-1 (ng/ml)	1.27 (0.67-2.17)	0.95 (0.42-1.46)**	1.01 (0.61-1.66)	0.67 (0.37-0.97)**
NAG (U/L)	8.5 (5.4-13.3)	7.8 (5.4-14.1)	2.0 (1.2-3.6)**	0.3 (0.2-0.3)**
Cystatin C (mg/L)	0.03 (0.01-0.05)	0.03 (0.02-0.05)	0.03 (0.01-0.05)	0.04 (0.02-0.06)**
NGAL (ng/ml)	34.2 (13.3-98.3)	32.4 (7.8-89.6)	18.3 (3.7-93.6)	18.3 (7.3-77.5)*
H-FABP (ng/ml)	1.56 (0.39-7.59)	1.50 (0.39-4.26)*	0.97 (0.39-4.64)	0.95 (0.39-4.05)

Data are shown as medians (interquartile range). $p<0.05$, ** $p < 0.001$ compared to fresh samples (Wilcoxon Signed Rank Test).

Change in renal damage markers after frozen storage

Table 1 shows the urinary concentrations of the various renal damage markers when measured in fresh urine samples and when measured at different time points after frozen storage at -80 ^{0}C. All markers, except cystatin C, showed a gradual decline in median concentration after frozen storage, although not all values reached statistical significance. Most outspoken was the decline in urinary NAG concentration, which decreased 28-fold after frozen storage during 1 year.

Variability in renal damage markers after frozen storage

Figure 1 shows for each renal damage marker the change (median and interquartile ranges) in concentration from values measured in fresh urine samples when a. using the same fresh urine sample and the same assay (representing intra-assay variation), b. using the same fresh urine sample but another assay (representing inter-assay variation), c. after 1 week, d. after 6 months and e. after 1 year of frozen storage at -80 ^{0}C. These figures show that in general the interquartile range after frozen storage is wider than when concentrations are measured in fresh urine samples, indicating that frozen storage induces an increase in variability for all measured markers. In

general, however, these interquartile ranges do not become wider after longer storage.

Effect of various storage protocols

We tested whether different storage protocols could minimize the average decline in urinary renal damage markers observed after frozen storage during 1 year at -80^{0}C. Results are shown in table 2. In general, none of the investigated storage protocols could prevent this decline.

Correlation to fresh samples

Table 3 shows the R square values of the correlations and Bland Altman agreements between urinary damage markers when assessed in fresh urine samples and when assessed after 1 year storage at -80 or -20 ^{0}C in the same samples using various storage protocols. This table shows that the concentrations of all markers using all storage protocols are significantly correlated to the freshly measured concentrations and that agreement is not fundamentally improved by different storage protocols. In general the various storage protocols (primary alkalinisation, secondary alkalinisation and adding protease inhibitors) did not result in relevantly higher R square values or smaller Bland Altman agreements when compared to the situation when no specific storage protocol was followed.

Table 2 Concentration of various renal damage markers when measured in fresh urine samples (n=95) and in the same urine samples after 1 year frozen storage at -80 and -20 ºC using various storage protocols.

		-80 ^{0}C				-20 ^{0}C	
	fresh	*no storage protocol*	*primary alkalinisation*	*secondary alkalinisation*	*protease inhibitors*	*no storage protocol*	*secondary alkalized*
IgG-total (µg/ml)	6.4 (2.1-28.9)	5.0 (2.2-15.3)	3.8 (1.4-9.1)$^{\#\#}$	2.9 (0.1-12.0)	3.9 (0.1-11.5)	1.0 (0.1-15.1)	3.6 (2.6-7.3)
IgG-4 (µg/ml)	1.2 (0.9-2.6)	0.7 (0.3-2.9)	0.6 (0.4-1.8)$^{*\#}$	0.4 (0.02-1.6)$^{**\#\#}$	0.1 (0.02-0.7)$^{**\#\#}$	0.7 (0.4-2.0)*	0.7 (0.3-3.5)**
KIM-1 (ng/ml)	1.27 (0.67-2.17)	0.67 (0.37-0.97)**	0.70 (0.34-1.03)**	0.25 (0.04-0.52)$^{**\#\#}$	0.12 (0.08-0.16)$^{**\#\#}$	0.35 (0.19-0.74)$^{**\#\#}$	0.26 (0.06-0.48)$^{**\#\#}$
NAG (U/L)	8.5 (5.4-13.3)	0.28 (0.24-0.32)**	0.27 (0.25-0.32)**	0.22 (0.00-0.25)$^{**\#\#}$	0.28 (0.26-0.33)$^{**\#}$	0.27 (0.22-0.32)**	0.21 (0.00-0.24)$^{**\#\#}$
Cystatin C (mg/L)	0.03 (0.01-0.05)	0.04 (0.02-0.06)**	0.04 (0.03-0.06)**	0.03 (0.02-0.05)$^{**\#\#}$	0.03 (0.02-0.05)$^{**\#\#}$	0.00 (0.00-0.02)$^{**\#\#}$	0.02 (0.01-0.04)$^{**\#\#}$
NGAL (ng/ml)	34.2 (13.3-98.3)	18.3 (7.3-77.5)*	14.2 (9.7-78.2)$^{**\#\#}$	10.3 (4.6-62.4)$^{\#\#}$	17.7 (5.2-56.9)$^{**\#\#}$	21.9 (7.5-104.0)$^{\#\#}$	10.7 (0.0-44.8)$^{**\#\#}$
H-FABP (ng/ml)	1.56 (0.39-7.59)	0.95 (0.39-4.05)	1.15 (0.49-3.4)	0.83 (0.22-2.60)$^{**\#\#}$	0.89 (0.20-2.87)$^{**\#\#}$	1.04 (0.38-2.82)$^{**\#\#}$	0.86 (0.01-2.76)$^{**\#\#}$

Data are shown as medians (interquartile range). * p<0.05, ** p<0.01 compared to fresh samples (Wilcoxon Signed Rank Test). # p<0.05, ## p<0.01 compared to -80 ^{0}C 1 year storage (Wilcoxon Signed Rank Test)

Table 3 Relation between urinary damage markers when assessed in fresh urine samples and when assessed after 1 year storage at -80 or -20 ^{0}C using various storage protocols (same samples)

		-80 ºC				-20 ºC	
	fresh	*no storage protocol*	*primary alkalinisation*	*protease inhibitor*	*secondary alkalinisation*	*no storage protocol*	*secondary alkalinisation*
IgG-total	NA	0.46**	0.34**	0.31**	0.40**	0.20**	0.27**
IgG-4	NA	0.16**	0.21*	0.16**	0.12*	0.38**	0.45**
KIM-1	NA	0.54**	0.66**	0.44**	0.50**	0.21**	0.27**
NAG	NA	0.21**	0.16**	0.20**	0.15**	0.27**	0.19**
Cystatin C	NA	0.84**	0.92**	0.95**	0.94**	0.81**	0.07*
NGAL	NA	0.44**	0.48**	0.40**	0.44**	0.53**	0.57**
H-FABP	NA	0.54**	0.33**	0.54**	0.39**	0.30**	0.35**
IgG-total	NA	-0.18 (0.80)	-0.34 (0.87)	-0.75 (1.26)	-0.79 (1.25)	-0.71 (1.41)	-0.08 (1.03)
IgG-4	NA	-0.29 (1.11)	-0.45 (1.09)	-1.11 (1.09)	-0.88 (1.19)	-0.30 (1.05)	-0.43 (1.23)
KIM-1	NA	-0.46 (0.54)	-0.54 (0.49)	-1.49 (0.40)	-1.27 (0.52)	-0.92 (0.67)	-1.30 (0.71)
NAG	NA	-1.81 (0.30)	-1.80 (0.30)	-1.80 (0.31)	-1.83 (0.29)	-1.81 (0.28)	-1.85 (0.25)
Cystatin C	NA	0.39 (0.51)	0.41 (0.48)	0.24 (0.53)	0.31 (0.48)	-1.22 (0.84)	-0.41 (0.86)
NGAL	NA	-0.13 (0.94)	-0.31 (1.03)	-0.36 (0.98)	-0.67 (1.07)	-0.03 (1.00)	-1.09 (0.89)
H-FABP	NA	-0.25 (0.87)	-0.36 (1.03)	-0.18 (5.43)	-0.59 (0.97)	-0.49 (0.98)	-1.05 (1.39)

Upper panel shows R square values, and lower panel agreement according to Bland Altman, mean (SD). Abbreviations: NA, not applicable. * p<0.05, ** p < 0.001, Wilcoxon Signed Rank Test.

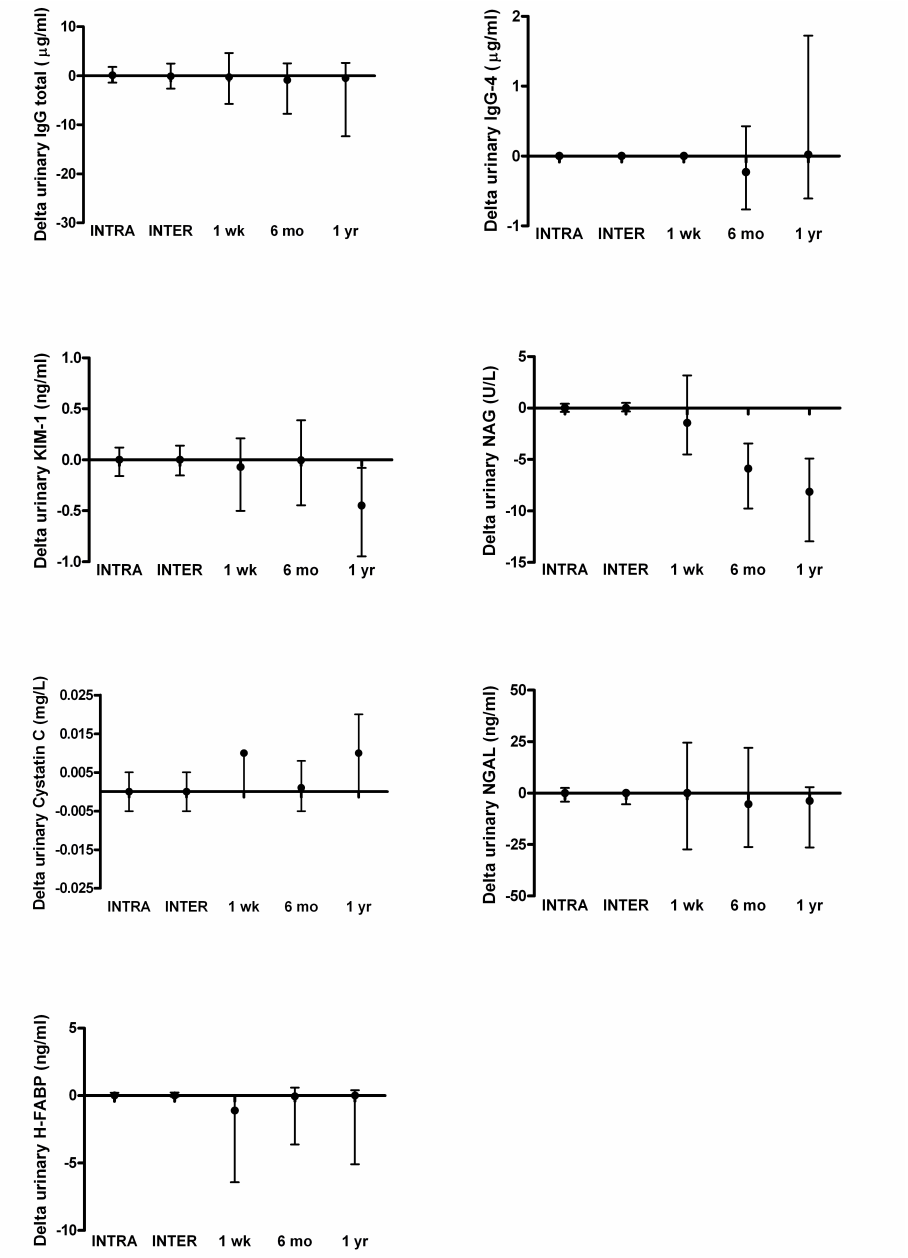

Figure 1 Absolute change in concentration of various urinary renal damage markers. INTRA means the difference between 2 samples measured in one run in the same assay, INTER means the difference between 2 samples measured using different assays, 1 wk, 6 mo and 1 yr mean the difference between concentrations assessed after frozen storage at -80º c in fresh samples and in samples stored during 1 week, 6 months and 1 year.

Sensitivity analyses

When all aforementioned investigations were redone with markers subdivided according to being under or above the median value measured in fresh urine samples essentially similar results were obtained in the high versus low marker concentration groups.

Discussion

In the present study, we investigated the influence of frozen storage on the concentration and variability of various urinary renal damage markers. We found that all markers, except cystatin C, show a decrease. Already after 1 week of frozen storage at -80 ºC remeasurement resulted in lower concentrations. Furthermore, all markers show an increase in variability after remeasurement after frozen storage. None of the investigated storage protocols could prevent or reduce these effects. Importantly, however, after frozen storage all markers remained significantly associated with values obtained when measured in fresh urine samples. No fundamental differences were observed when these investigations were done in samples stored at -20 ºC when compared to storage at -80 ºC.

We previously showed that 8 months of storage at -20 ºC results in a decrease in urinary albumin concentration of approximately 30%[5]. Only a limited number of studies has investigated the effect on the renal damage markers studied in the present protocol when measured in urine samples after frozen storage in comparison to when measured in fresh urine samples. First, in line with our findings Grenier et al reported that urinary NGAL concentrations declined significantly after 6 months storage at -20º C.[11] In contrast to our results, however, they found that NGAL concentrations did not decrease significantly when stored at -80°C. Second, Chaturvedi et al found no decline in KIM-1 concentration after 14 days of storage at -80º C as well as at -20º C[12], whereas we did observe a decrease. This may be due to the short duration of storage in this study, since we found a significant decline in urinary KIM-1 concentration especially after prolonged storage.

Third, Herget-Rosenthal et al found no decline in urinary cystatin C after frozen storage during 7 days,[13] which is in agreement with our data.

The results we observed were rather uniform, with two exceptions. Average urinary cystatin C concentration after frozen storage was nearly similar to the average concentration in fresh urine samples, even being slightly, but significantly higher, with a very accurate association between concentrations in fresh and frozen urine samples. On the other hand, average NAG concentrations declined strikingly in our study, both at -20º C as well as at -80º C. A reason for the decline in NAG concentration could be that we used an enzymatic assay that needs intact NAG to convert a substrate. However, no important differences have been described in NAG concentrations after frozen storage between measurement with an ELISA and an enzymatic assay.[14] Another explanation could be that the enzymatic activity of NAG decreases during storage due to pH effects, since it has been described that NAG isoenzyme A, which constitutes 80-90% of total NAG activity, is deactivated after 8 hours at pH >7 up to 40% and at pH >8 up to 100%.[14] However, mean urinary pH in our samples was 5.8, and only 5 out of our 95 samples had a pH > 7. Such a pH effect is therefore not a likely explanation for the degradation in NAG concentrations that we found.

The studies that previously investigated the effect of frozen storage on urinary renal damage marker concentration did not systematically study possible increases in variability, although one showed graphically that NGAL concentration showed higher variability after frozen storage at 20º C.[11] Our data indicate that all investigated markers, except cystatin C, increase in variability already after 1 week of frozen storage compared to their intra- and inter-assay variability assessed in fresh samples.

Why marker concentrations in general decline and show increased variability after frozen storage is unknown. Theoretically it may be that the molecules are trapped in the precipitate because of an alteration in pH of the urine

samples during freezing and thawing[15-17] or that bacterial contamination causing hydrolysis by bacterial proteases affects marker concentrations.[18] Furthermore, confirmational changes that occur during freezing or thawing may lead to loss of the antibody recognition site of the ELISA. To address the question why marker concentrations decline during frozen storage, we stored our urine samples under different conditions. Alkalizing urine samples or adding protease-inhibitors for instance, may be expected to prevent precipitate formation and hydrolysis due to bacterial proteases. Secondary alkalinisation might reverse a possible conformational change that could have occurred during freezing or thawing, or dissolve the investigated protein in case of entrapment in a precipitate. Since all these procedures did not prevent the decline in marker concentration during frozen storage, nor did they reduce variability to a substantial amount, no conclusions can be drawn from our results on the cause of the reduction in urinary biomarker concentration.

Should our data be interpreted that urinary biomarkers can not be assessed from urine specimens after prolonged frozen storage? In our opinion this is not the case. Although after frozen storage these markers show on average a reduction and an increase in variability, our data also show that in general the situation after 1 year of storage is not fundamentally different when compared to situation after 1 month of storage. Moreover, measurement after 1 year yields significant correlations with values measured in fresh urine samples. We therefore conclude that urinary biomarkers should be measured preferably in fresh urine samples, but measurement in urine samples that have been stored at -80º C or -20º C is acceptable for most markers. However, our data indicate that results from studies that for instance measure biomarkers from frozen urine samples as potential predictors for clinical outcome should be interpreted with caution. Absence of predictive value could be due to the fact that the marker under investigation indeed has no predictive value, but could also be due to the fact that the decline or variability that is induced by frozen storage has obscured the

relation that in reality was present. Previously we have shown this principle for urinary albumin concentration.[8]

We acknowledge that our study has limitations. First, all marker concentrations have been measured with 1 assay only. It can not be excluded that other assays with antibodies directed against other epitopes will yield other results. However, since all markers that we investigated showed more or less the same pattern it is unlikely in our opinion that different conclusions will be reached using other assays. Second, damage markers were determined in urine samples of patients with CKD. Damage marker concentrations are thus relatively low when compared to the situation in urine samples of patients with acute kidney injury. Storage effects might be different in a higher concentration range. However, we found within the range of concentrations we measured no essentially different results between low and high concentrations. Third, in urine samples of patients with diabetes certain constituents might play a role in the decline of marker concentrations that are not present in urine samples of patients with other kidney diseases (or vice versa).

Strengths of this study are that we measured multiple markers in a large number of urine samples, investigating two storage temperatures and the time course of changes in marker concentration. Other studies investigating stability of tubular damage markers have measured maximally 1-3 markers[11;13;19], in a limited number of samples.[19] Furthermore, we tried to identify possible mechanisms of loss of concentration by investigating whether specific storage conditions result in more reliable results after frozen storage.

In conclusion, based on our observations we recommend that urinary renal damage markers should preferably be measured in fresh urine samples. If this is not possible, they can be stored at -80º C or -20º C. Frozen storage is, however, associated with a reduction in average value and an increase in

variability of most markers. Studies using frozen urine samples should therefore be interpreted with caution.

Acknowledgements

We thank Jan Roggeveld and Betttine Haandrikman for their technical assistance.

Statement of competing financial interests

None of the authors has anything to declare.

Reference List

1. Coca SG, Yalavarthy R, Concato J, Parikh CR. Biomarkers for the diagnosis and risk stratification of acute kidney injury: a systematic review. *Kidney Int* 2008; 73: 1008-1016

2. Damman K, van Veldhuisen DJ, Navis G, Voors AA, Hillege HL. Urinary neutrophil gelatinase associated lipocalin (NGAL), a marker of tubular damage, is increased in patients with chronic heart failure. *Eur J Heart Fail* 2008; 10: 997-1000

3. Meijer E, Boertien WE, Nauta FL *et al*. Association of urinary biomarkers with disease severity in patients with autosomal dominant polycystic kidney disease: a cross-sectional analysis. *Am J Kidney Dis* 2010; 56: 883-895

4. Nauta FL, Boertien WE, Bakker SJ *et al*. Glomerular and Tubular Damage Markers Are Elevated in Patients With Diabetes. *Diabetes Care* 2011;

5. Brinkman JW, de Zeeuw D, Duker JJ *et al*. Falsely low urinary albumin concentrations after prolonged frozen storage of urine samples. *Clin Chem* 2005; 51: 2181-2183

6. Brinkman JW, de Zeeuw D, Lambers Heerspink HJ *et al*. Apparent loss of urinary albumin during long-term frozen storage: HPLC vs immunonephelometry. *Clin Chem* 2007; 53: 1520-1526

7. Lambers Heerspink HJ, Nauta FL, van der Zee CP *et al*. Alkalinization of urine samples preserves albumin concentrations during prolonged frozen storage in patients with diabetes mellitus. *Diabet Med* 2009; 26: 556-559

8. Brinkman JW, de Zeeuw D, Gansevoort RT *et al*. Prolonged frozen storage of urine reduces the value of albuminuria for mortality prediction. *Clin Chem* 2007; 53: 153-154

9. Zhou H, Yuen PS, Pisitkun T *et al*. Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. *Kidney Int* 2006; 69: 1471-1476

10. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986; 1: 307-310

11. Grenier FC, Ali S, Syed H *et al*. Evaluation of the ARCHITECT urine NGAL assay: assay performance, specimen handling requirements and biological variability. *Clin Biochem* 2010; 43: 615-620

12. Chaturvedi S, Farmer T, Kapke GF. Assay validation for KIM-1: human urinary renal dysfunction biomarker. *Int J Biol Sci* 2009; 5: 128-134

13. Herget-Rosenthal S, Feldkamp T, Volbracht L, Kribben A. Measurement of urinary cystatin C by particle-enhanced nephelometric immunoassay: precision, interferences, stability and reference range. *Ann Clin Biochem* 2004; 41: 111-118

14. Morita A, Numata Y, Kosugi Y, Noto A, Takeuchi N, Uchida K. Stabilities of N-acetyl-beta-D-glucosaminidase (NAG) isoenzymes in urine: advantage of NAG isoenzyme B measurement in clinical applications. *Clin Chim Acta* 1998; 278: 35-43

15. Townsend JC. Effect of storage temperature on the precipitation of albumin from urine. *Clin Chem* 1986; 32: 1986-1987

16. Townsend JC, Sadler WA, Shanks GM. The effect of storage pH on the precipitation of proteins in deep frozen urine samples. *Ann Clin Biochem* 1987; 24 (Pt 1): 111-112

17. Saetun P, Semangoen T, Thongboonkerd V. Characterizations of urinary sediments precipitated after freezing and their effects on urinary protein and chemical analyses. *Am J Physiol Renal Physiol* 2009; 296: F1346-F1354

18. Rowe DJ, Dawnay A, Watts GF. Microalbuminuria in diabetes mellitus: review and recommendations for the measurement of albumin in urine. *Ann Clin Biochem* 1990; 27 (Pt 4): 297-312

19. Han WK, Wagener G, Zhu Y, Wang S, Lee HT. Urinary biomarkers in the early detection of acute kidney injury after cardiac surgery. *Clin J Am Soc Nephrol* 2009; 4: 873-882

Chapter 5

Albuminuria, proteinuria and novel urine biomarkers as predictors of long term allograft outcomes in kidney transplant recipients

American Journal of Kidney Disease, 57: 733-743, 2011

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Abstract

Proteinuria is an established marker of chronic renal transplant dysfunction. Recently it has been suggested that albuminuria might be a more reliable marker for allograft injury. Albuminuria is often regarded as a marker of glomerular damage. However, since chronic renal allograft injury is thought to be predominantly an interstitial process, albuminuria might in this case reflect tubular damage. We aimed to study the value of albuminuria, proteinuria and tubular damage markers (KIM-1, NAG, NGAL, H-FABP) predicting graft outcome in a cohort of renal transplant recipients.

606 patients visiting our renal transplant outpatient clinic between 2001 and 2003 were included and were used in the analysis for death censored graft failure. Median follow-up was 4.7 (3.8-5.2) year. 577 patients had follow-up >1 year and were included the analysis for renal function decline. Median follow-up was 3.2 (2.7-3.7)

We have measured urinary protein, albumin and specific tubular damage markers in 24h-urine samples. Primary outcomes were occurrence of death censored graft failure and renal function decline. 42 patients developed graft failure and mean change in renal function was -0.46 (3.7) ml/min per year. ROC-curve analysis revealed that albuminuria has the highest area under the curve (95%CI) in predicting death-censored graft failure (0.78, 0.59-0.76), significantly higher than proteinuria (0.67, 0.59-0.76; p= 0.001), NGAL (0.63, 0.52-0.74; p=0.02) and H-FABP (0.62 0.53-0.73; p=0.005), but not different than the area under the curve of KIM-1 and NAG excretion.

We conclude that measuring albuminuria to predict long-term renal outcome in renal transplant recipients may be superior to measuring total proteinuria. Additional assessment of the urinary excretion of tubular damage markers has limited value to predict outcome.

Introduction

One year graft survival after kidney transplantation has greatly improved over the years. In contrast, long-term graft survival has changed only to a limited extent. Approximately half of all deceased renal allografts are still lost within 10 to 12 years after transplantation.[1] One of the leading causes of late allograft loss is Chronic Transplant Dysfunction[2] , which is characterised by decline in renal function with hypertension and proteinuria.[3;4] This is accompanied by tubular histological changes as interstitial fibrosis and tubular atrophy. [5;6]

There is a need for markers that can predict the initiation and progression of chronic transplant dysfunction in an early phase. [7] This would enable intervention or change in therapy. In clinical practice estimates of glomerular filtration rate and proteinuria are used for identification of transplant recipients at increased risk for late renal allograft loss. [8-10] Proteinuria results from the urinary loss of various proteins, whereas albuminuria is less heterogenic. Interestingly, it has recently been suggested that albuminuria may be a more reliable marker of chronic renal allograft injury than proteinuria.[11] Although albuminuria may be less heterogenic than proteinuria, it still is the resultant of two counteracting processes, glomerular leakage and tubular reabsorption. Since chronic transplant dysfunction is predominantly an interstitial process, it might be that in renal transplant recipients specific tubular damage markers, such as Kidney Injury Molecule 1 (KIM-1) or Neutrophil Gelatinase-Associated Lipocalin (NGAL), are better predictors for renal function outcome than albuminuria. We investigated as proximal tubular markers KIM-1, NAG (N-Acetyl-beta-D-Glucosaminidase) and NGAL because these markers have different mechanisms of release and represent a different kind of tubular damage, and because in recent years they are extensively being investigated. As distal tubular marker we investigated H-FABP (Heart Fatty Acid Binding Protein) since it is a sensitive and rapid new marker of distal tubular injury.[12]

Given this background, we studied whether albuminuria is superior to proteinuria in predicting graft failure and change in renal function in renal transplant recipients. Also, we aimed to investigate whether specific tubular damage markers predict change in renal function and graft failure, and whether these markers add to the predictive value of albuminuria.

Methods

Study design and patients

After a baseline visit all participating subjects visited the out-patient clinic at least once a year. Current medication was taken from the medical record. Details of this study, among others standard immunosuppressive treatment, have been described previously.[13] Excluded from our study were recipients with missing samples at baseline (n=8). The Institutional Review Board approved the study protocol (METc 01/039) which was in adherence to the declaration of Helsinki. Funding sources had neither a role in the collection and analysis of data, nor in the submission and publication of the manuscript.

Definition of endpoints

The primary endpoints of this study were death censored graft failure and change in renal function. Death censored graft failure was defined as return to dialysis or retransplantation. Change in renal function was defined as eGFR at baseline subtracted by eGFR at the last follow-up visit, divided by time from baseline to follow up, and expressed in ml/min*1.73m^2 per year. This is preferred to a slope since renal function decline in CTD does usually not happen in a linear manner.[14] Calculating a slope instead of a change in eGFR will therefore underestimate a patient's true renal function decline.

For patients who died with a functioning graft (n=78) eGFR at follow-up was defined as renal function at the last visit to the out-patient clinic prior to death, and for patients with graft failure (n = 42) eGFR at follow-up was

defined as the eGFR value at the last visit to the out-patient clinic before starting dialysis or retransplantation. There was no loss to follow-up.

Measurements

Serum creatinine levels were determined using the Jaffé method (MEGA AU 510, Merck Diagnostica, http://www.merck.de/en/index.html). Urinary creatinine excretion was normalized to body weight by dividing the 24h urinary creatinine excretion by the patient's weight in kilograms. GFR was estimated with the 4-variable MDRD formula: we have previously documented the validity of this formula to estimate GFR in our transplant population[15] At baseline all participating subjects collected a 24 hr urine. After collection, these 24-hour urinary samples were centrifuged. A sample was used for routine measurements such as creatinine, and was immediately stored (-80°C) in plastic aliquots until measurement. No protease inhibitors were added. After thawing all urine samples were vortexed and subsequently centrifuged (14.000 rpm). The supernatant was used for measurements. All urinary biomarkers were determined from these frozen samples in one run.

Total protein concentration was analysed using the pyrogallol red-molybdate method (Merck Dianostica,MEGA, http://www.merck.de/en/index.html, intra-assay CV 14%). Urinary albumin was measured by nephelometry (Dade Behring Nephelometer, intra-assay CV 2.7%, http://www.dadebehring.com/edbna2/ebusiness/home.jsp). For biomarker quantification in urine we developed direct sandwich-enzyme-linked immunosorbent assays (ELISA's) using monoclonal coating antibodies (MAb) and labeled polyclonal detection antibodies on a Maxisorp plate (Nunc, http://www.nuncbrand.com/en/default.aspx) in which is the concentration of the analyte was determined spectrophotometrically by conversion of *o*-phenylenediamine by Horse-Radish Peroxidase label. Samples were diluted before measurement (2 times for KIM-1, 100 times for NGAL, 5 times for HFABP). KIM-1 and NGAL antibodies were obtained from R&D systems (http://www.rndsystems.com).

H-FABP antibodies were obtained from Hytest http://www.hytest.fi). The intra-assay coefficients of variation were 7.4%, 6.8% and 9.3%, respectively. The lower limits of detection were 1.0 (ng/ml), 2.5 (ng/ml), and 3.0 respectively. Urinary concentration of NAG was measured using a modified enzyme assay according to Lockwood and corrected for nonspecific conversion (HaemoScan, http://haemoscan.com, intra-assay CV 3.1%). The lower limit of detection was 2.7 (U/L). Urinary biomarker excretion is expressed per 24 hours.

Statistical analysis

Analyses were performed with SPSS version 16.0 (SPSS Inc., Chicago, IL). Parametric variables are expressed as mean ± standard deviation (SD), whereas non-parametric variables are given as median (interquartile range). A two sided $p < 0.05$ was considered to indicate statistical significance.

To investigate the predictive value of proteinuria, albuminuria and tubular damage markers at baseline for death censored graft failure, various analyses were performed. To visualise the associations between these urinary biomarkers and graft failure, we divided our cohort into quartiles of increasing urinary excretion of each biomarker. These quartiles were plotted against the incidence of graft failure (figure 1). Furthermore, ROC curves were drawn for each biomarker (figure 2). Calculation of the statistical significance between the various ROC curves was performed using Stata version 10.1 software (StataCorp, Texas, USA) using time independent C-statistics.

Lastly, Cox regression analyses were performed to investigate whether these urinary biomarkers at baseline are independently associated with graft-failure during follow-up (table 3). First, these analyses were performed crude. Second, multivariate regression analyses were performed, adjusting for age, sex, and baseline eGFR. Third, we tested whether information on the urinary excretion of the various tubular damage markers adds to the

predictive value of albuminuria by implementing both albuminuria and the urinary excretion of the biomarker under investigation in the model. Hazard ratios (HR) are reported with 95% confidence interval. As secondary analyses we investigated the composite of graft failure and doubling of serum creatinine and the composite of doubling of serum creatinine, kidney failure and death. These outcomes being of clinical interest and offering the advantage of enhancing power by including more end-points.

To investigate the predictive value of proteinuria, albuminuria and tubular damage markers at baseline for change in renal function during follow-up, patients with a follow-up < 1 year were excluded from the analysis (n=29). We again divided our cohort into quartiles of increasing urinary excretion of each biomarker, and these quartiles were plotted against change in renal function during follow-up (figure 3). Furthermore, linear regression analyses were performed (table 4) with baseline urinary biomarker excretion as independent and change in renal function as dependent variable. Second, this association was adjusted for gender, age, and baseline eGFR. Third, we tested whether tubular damage markers add to the predictive value of albuminuria by implementing both albuminuria and the urinary excretion of the biomarker in the model.

Biomarker excretion was $\log_2$-normalised to fulfil the requirements for linear regression analysis. These log transformed values have been standardized to enable comparison between markers. We tested for significant interactions between patient characteristics and baseline urinary biomarker excretion by entering the urinary biomarker, the investigated characteristic, and their product term in the same multivariate regression model.

Results

In this prospective longitudinal study, all renal transplant recipients were eligible who visited our out-patient clinic between August 2001 and July 2003 and had a functioning graft for at least 1 year. Patients with known or

apparent systemic illnesses (e.g. malignancies or opportunistic infections) were considered ineligible. A total of 606 out of 847 (72%) subjects signed written informed consent. [16] (Figure 1) Recipients participated at a median of 5.9 (2.6-11.4) years post transplant in baseline measurements. Follow-up time for graft failure beyond baseline was 4.7 (3.8-5.2) years and for change in renal function 3.2 (2.7-3.7) years. The number of patients that developed graft failure was 42 and the mean change in renal function was -0.46 (3.7) ml/min per year. Patient characteristics are presented in table 1 stratified for albuminuria. All endpoints and their composites are shown in table 2. Urinary creatinine excretion normalized to body weight was 17.7 ± 4.6 mg/kg. Median proteinuria was 568 (340-872) mg/24h and albuminuria 40 (15-170) mg/24h. Median biomarker excretions are also presented in table 1 and their distribution is shown graphically in figure 2. Correlations between all markers are shown in supplementary table 1.

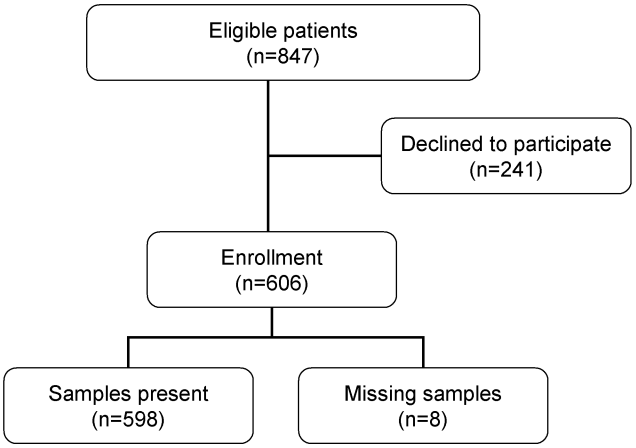

Figure 1 Flow diagram shows formation of the cohort

Graft failure

Rates of graft failure during follow-up according to quartiles of 24hr urinary excretion of the various biomarkers under study are depicted in figure 3. This figure shows clear associations between 24hr excretions of total protein excretion, albumin, KIM-1 and NAG versus incidence of death censored graft failure, whereas the association between baseline excretion of H-FABP and NGAL versus incident graft failure is less clear.

ROC curves for urinary biomarker excretion predicting graft failure are shown in figure 4. The areas under the ROC curve (95%CI) for urinary excretion of total protein, albumin, KIM-1, NAG, H-FABP and NGAL were 0.67 (0.59-0.76; $p<0.001$), 0.78 (0.70-0.87; $p<0.001$), 0.74 (0.66-0.82; $p<0.001$), 0.75 (0.67-0.83; $p<0.001$), 0.62 (0.53-0.72; $p=0.009$) and 0.63 (0.53-0.74; $p=0.007$) resp. Albuminuria has the highest AUC in predicting graft failure. The AUC's for the predictive value of urinary excretion of total protein, H-FABP and NGAL were significantly lower ($p=0.001$, $p=0.005$ and $p=0.02$, respectively) than for albuminuria. The AUC's for the predictive value of KIM-1 and NAG did not differ significantly from albuminuria ($p=0.19$, $p=0.19$ resp.).

Table 1 Patient characteristics in albuminuria strata

Variable	*(<10 mg/24h)*	*10-30 mg/24h)*	*(30-300 mg/24h)*	*(>300 mg/24h)*
	N=96	*N=159*	*N=237*	*N=106*
Age (years)	53 (44-60)	53 (42-61)	53 (44-62)	50 (41-58)
Male gender (%)	45	49	57	62
Current smoking (%)	19	22	20	30
BMI (kg/m^2)	26.1 (4.6)	26.3 (4.3)	26.0 (4.2)	25.8 (4.4)
MAP (mm Hg)	107 (13)	108 (13)	112 (12)	116 (13)
Plasma cholesterol (mg/dL)	220 (197-236)	213 (189-236)	217 (189-240)	224 (193-251)
Time after transplantation (years)	5.1 (2.2-11.6)	4.7 (1.7-10.6)	6.0 (2.8-10.7)	9.1 (5.4-14.0)
Time on dialysis prior to transpantation (years)	2.6 (1.3-3.9)	2.2 (0.9-4.0)	2.3 (16-49)	1.9 (0.8-4.1)
Living donation (%)	13	17	14	11
Donor age *(year)*	32 (19-45)	36 (23-48)	41 (25-53)	34 (22-49)
Calcineurin inhibitors	83	81	79	69
RAAS-inhibition (%)	35	26	33	42
eGFR at baseline (MDRD) ($ml/min* 1.73m^2$)	51 (13)	50 (14)	45 (14)	41 (15)
- Proteinuria (mg/24h)	395 (259-621)	410 (288-659)	568 (370-789)	1100 (710-1512)
- Albuminuria (mg/24h)	7 (5-8)	18 (13-24)	90 (47-144)	572 (409-1031)
Proximal tubular markers				
- KIM-1 (µg/24h)	0.6 (0.2-1.1)	0.7 (0.3-1.3)	1.6 (0.9-2.3)	2.4 (1.4-3.4)
- NGAL (µg/24h)	281 (227-358)	320 (249-409)	339 (270-491)	353 (254-490)
- NAG (U/24h)	4.8 (2.6-9.7)	7.8 (4.1-12.1)	9.6 (6.1-15.2)	13.0 (8.8-20.4)
Distal tubular marker				
- H-FABP (µg/24h)	3.1 (1.6-5.8)	4.1 (2.1-7.8)	5.2 (2.5-10.8)	10.7 (3.8-23.2)
Creatinine excretion (g/24h)	1.33 (1.10-1.61)	1.32 (1.06-1.61)	1.31 (1.04-1.61)	1.36 (1.12-1.62)

N = 598. Parametric variables are expressed as mean ± SD, whereas non-parametric variables are given as median (25^{th}-75^{th} percentile). Abbreviations are: BMI, body mass index; MAP, Mean Arterial Pressure; eGFR, estimated Glomerular Filtration Rate; KIM-1, Kidney Injury Molecule 1; NGAL, Neutrophil Gelatinase-Associated Lipocalin; NAG, N-acetyl-beta-D-glucosaminidase; H-FABP, Heart Fatty Acid Binding Protein.

Table 2 Endpoints specified in albuminuria strata

Variable	*(<10 mg/24h)*	*10-30 mg/24h)*	*(30-300 mg/24h)*	*(>300 mg/24h)*
	N=96	*N=159*	*N=237*	*N=106*
Graft failure (death censored) (n)	2 (2.1%)	3 (1.9%)	11 (4.6%)	26 (24.5%)
Doubling of serum creatinine (n)	2 (2.1%)	1 (0.6%)	7 (3.0%)	4 (5.7%)
Composite of graft failure + doubling of serum creatinine (n)	4 (4.2%)	4 (2.5%)	13 (5.5%)	26 (24.5%)
All cause mortality (n)	7 (7.3%)	22 (13.8%)	40 (16.9%)	22 (20.8%)
Composite graft failure + doubling serum creatinine + ACM (n)	10 (10.4%)	25 (15.7%)	52 (21.9%)	40 (37.7%)
Follow-up (years)	4.6 (1.0)	4.4 (1.3)	4.3 (1.3)	3.7 (1.8)
Change in eGFR (ml/min per year)	-0.14 (2.71)	0.07 (4.23)	-0.42 (3.8)	-1.57 (2.89)

N=598. Various end points and the composites used in analyses are shown. Values shown as number, number (percentage), or mean ± standard deviation. Abbreviations: eGFR, estimated glomerular filtrations rate; ACM, all cause mortality.

The results of Cox regression analyses are given in table 3. These data show that univariate all urinary biomarkers predict graft failure (left columns). When adjusted for age, sex, and baseline eGFR NGAL and H-FABP loose statistical significance for predicting outcome, whereas KIM-1 and NAG pertain statistical significance (middle columns). When the various urinary biomarkers are forced into a model that also contains albuminuria, albuminuria remains a significant predictor of outcome, whereas none of the urinary biomarkers reach statistical significance (right columns). Also when adjustments are made for a large number of possible confounders (model 4) the results do not change materially. As secondary analyses we investigated the composite of graft failure and doubling of serum creatinine and the composite of doubling of serum creatinine, kidney failure and death (table 3). These analyses rendered the same conclusion. Urinary total protein, KIM-1 and NAG excretion are all significant independent predictors of these composite endpoint in addition to albuminuria. As second sensitivity analysis we have also calculated the damage marker to creatinine ratio to correct for potential urine collection errors. Analyses on graft failure provides similar results as 24-hour excretions (see supplementary table 2). Furthermore, when separate analyses are performed in normo-albuminuric patients (<30 mg/24h) or in patients with albuminuria <100 mg/24h, none of the investigated damage markers adds significantly to the predictive capacity of albuminuria (data not shown).

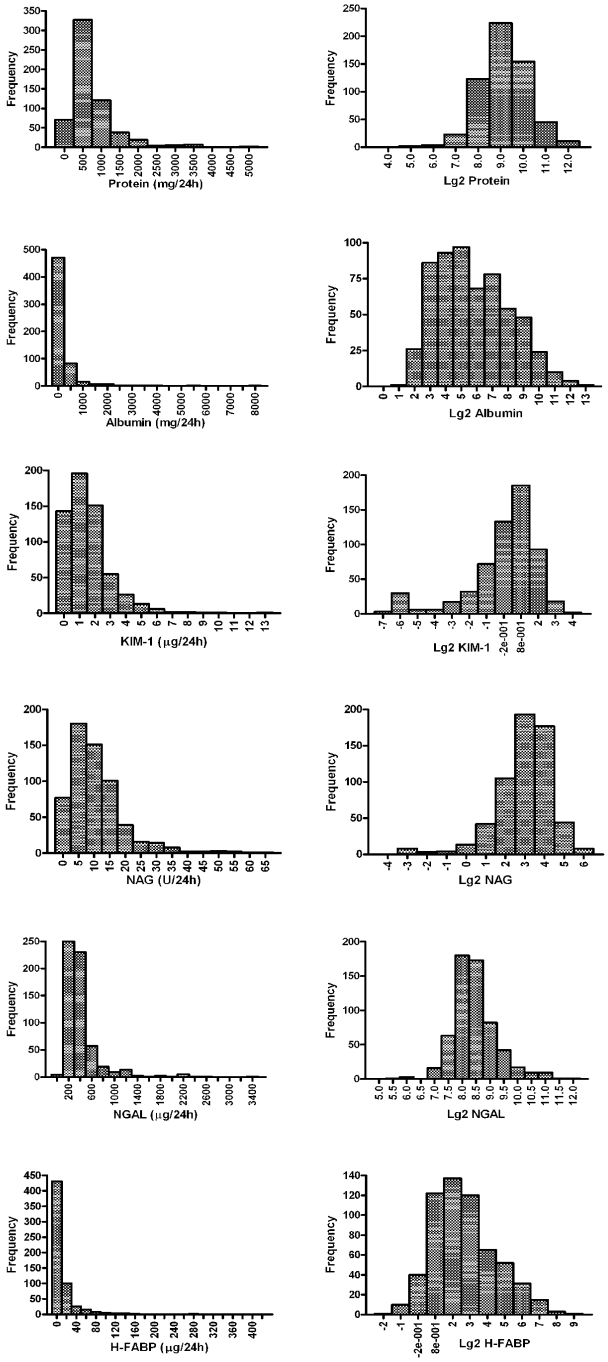

Figure 2 Distribution of markers

Table 4 Association between biomarkers at baseline and change in eGFR per year during follow-up

	Model 1		*Model 2*		*Model 3*			
	Biomarker (Std.β)	p-value	Biomarker (Std.β)	p-value	Biomarker (Std.β)	p-value	Albuminuria (Std.β)	p-value
- Proteinuria	-0.15 (-0.46; 0.16)	0.3	-0.18 (-0.49; 0.13)	0.3	0.13 (-0.22; 0.47)	0.5	-0.72 (-1.1; -0.35)	<0.001
- Albuminuria	-0.59 (-0.91; -0.27)	<0.001	-0.66 (-0.98; -0.33)	<0.001	NA	NA	NA	NA
Proximal tubular								
- KIM-1	-0.47 (-0.77; -0.17)	0.002	-0.54 (-0.84; -0.23)	0.001	-0.32 (-0.66; 0.02)	0.7	-0.51 (-0.87; -0.14)	0.006
- NAG	-0.21 (-0.52; 0.09)	0.2	-0.31 (-0.63; 0.01)	0.06	-0.16 (-0.49; 0.17)	0.3	-0.62 (-1.0; -0.29)	<0.001
- NGAL	-0.02 (-0.34; 0.30)	0.9	-0.17 (-0.50; 0.17)	0.3	-0.03 (-0.37; 0.32)	0.9	-0.65 (-0.98; -0.32)	<0.001
Distal tubular								
- H-FABP	-0.44 (-0.75; -0.13)	0.005	-0.50 (-0.82; -0.19)	0.002	-0.37 (-0.69; -0.04)	0.03	-0.58 (-0.91; -0.25)	0.001

	Model 4			
	Biomarker (Std.β)	p-value	Albuminuria (Std β)	p-value
- Proteinuria	0.14 (-0.21; 0.50)	0.4	-0.70 (-1.1; -0.31)	<0.001
- Albuminuria	-0.62 (-0.97; -0.28)	<0.001	NA	NA
Proximal tubular				
- KIM-1	-0.22 (-0.57; 0.13)	0.2	-0.52 (-0.90; -0.13)	0.008
- NAG	0.12 (-0.45; 0.22)	0.5	0.60 (-0.95; -0.24)	0.001
- NGAL	-0.03 (-0.37; 0.32)	0.9	-0.62 (-0.97; -0.26)	0.001
Distal tubular				
- H-FABP	-0.32 (-0.65; 0.01)	0.06	-0.56 (-0.91; -0.20)	0.002

Data are given as standardized β (95% CI) and p-values. Biomarker excretions are 2log transformed to fulfil requirements of linear regression analysis. Standardized β show increased risk on 1 ml/min/per year decrease in eGFR per two fold increase of biomarker excretion. Model 1: Crude model; model 2: Adjustment for age, sex and eGFR; model 3: Model 2 + albuminuria; Model 4: Model 3 + time between renal Tx and baseline, donor age, living related or cadaveric Tx, BMI, MAP, smoking, total cholesterol, duration warm ischemia time, duration cold ischemia time, acute rejections, use of calcineurin inhibitors, diabetes mellitus and daily dose of prednisolon at baseline.

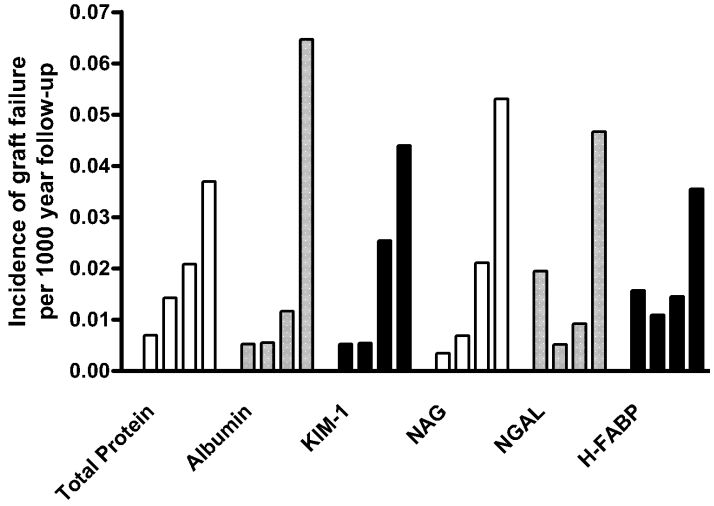

Figure 3 Quartiles of increasing urinary excretion of various biomarkers at baseline versus incidence of graft failure per 1.000 years of follow-up.

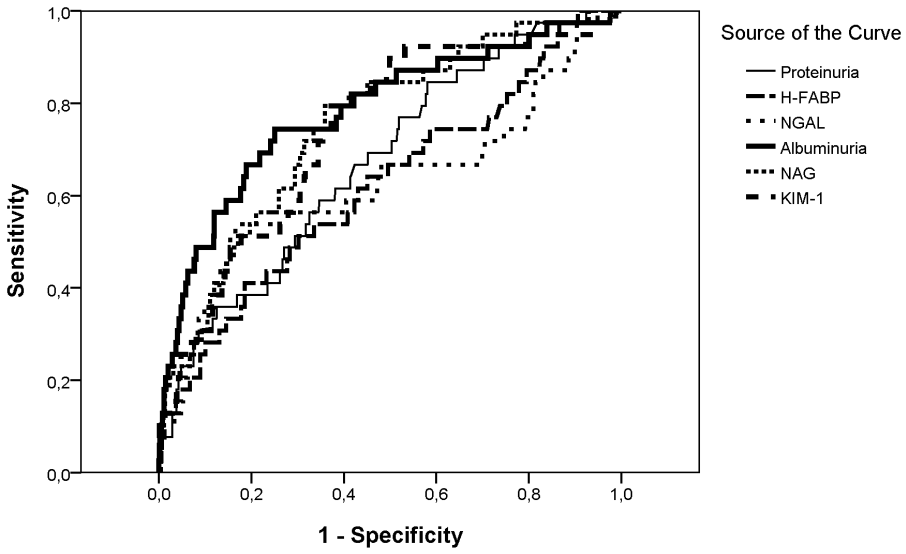

Figure 4 Areas under the curve

Change in renal function

Changes in renal function according to quartiles of 24hr urinary excretion of the various biomarkers under study are depicted in figure 5. This figure shows clear associations between 24hr excretions of albumin, KIM-1 and NAG versus change in renal function per year, whereas the association between baseline excretion of total protein H-FABP and NGAL versus change in renal function per year is less clear.

The results of linear regression analyses are given in table 4. These data show that univariately all urinary biomarkers but total protein, NGAL and NAG predict change in renal function (left column). When adjusted for age, sex, and baseline eGFR, the markers total protein, NGAL and NAG remain statistical unsignificant for predicting outcome (middle columns). When the various urinary biomarkers are forced into a model that also contains albuminuria, these data show that in all cases albuminuria remains a significant predictor of outcome, whereas of the urinary biomarkers only H-FABP remains a statistical significant predictor. To investigate the contribution of other known risk factors for graft failure to the predictive model we adjusted in a sensitivity analysis for a large number of potential confounders (model 4). This did not materially change the results. In all outcomes albuminuria remained significantly associated with change in renal function. As sensitivity analysis this analysis has also been repeated with damage marker to creatinine ratio's. This provided similar results (see supplementary table 3).

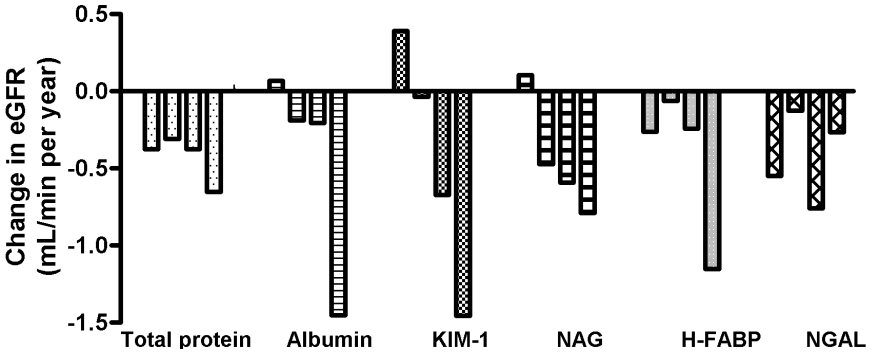

Figure 5 Quartiles of increasing urinary excretion of various biomarkers at baseline versus mean change in eGFR per year during follow-up. Cut-off values of quartiles are for proteinuria <340.3 mg/24h, 340.3-568.0 mg/24h, 568.0-871.0 mg/24h and > 871.0 mg/24h; for albuminuria <14.5 mg/24h, 14.5-40.4 mg/24h, 40.4-169.7 mg/24h and >169.7 mg/24h; for KIM-1 <0.52 µg/24h, 0.52-1.22 µg/24h, 1.22-2.13 µg/24h and >2.13 µg/24h; for NGAL <254 µg/24h, 254-321µg/24h, 321-443 µg/24h and >443 µg/24h; for NAG <4.8 U/24h, 4.8-9.0 U/24h, 9.0-14.8 and >14.8 U/24h; for H-FABP <2.3 µg/24h, 2.3-4.8 µg/24h, 4.8-12.0 µg/24h and >12.0 µg/24h.

Discussion

In this longitudinal prospective study, we found that albuminuria is a powerful independent risk marker for both graft failure and change in renal function during follow-up. Albuminuria outperforms proteinuria with respect to renal prognosis. Furthermore, two out of four investigated tubular damage markers (KIM-1 and NAG) predict graft failure and kidney function loss, however, not independent from albuminuria.

With respect to our primary endpoint we found that all investigated tubular damage markers predict death censored graft failure when considered separately. These markers have predominantly been investigated for the prediction of acute kidney injury and acute rejection.[17-19] Little is known about tubular biomarkers beyond the first year after transplantation.

KIM-1 is a transmembrane immunoglobulin, which is undetectable in normal kidneys, but markedly upregulated in damaged tubular epithelial cells after different types of renal injury[20-22] Urinary KIM-1 levels correlate to intrarenal KIM-1 expression[23], and elevated urinary KIM-1 decreases upon resolution of renal damage and renoprotective intervention[24]. Our data are in

agreement with an earlier study showing that the urinary excretion of KIM-1 related to the extent of renal damage in various renal diseases.[25] In a cohort of 145 kidney transplant patients we previously reported that KIM-1 is a powerful predictor of graft failure.[26] In this larger cohort we corroborate that KIM-1 is a predictor of graft failure (HR 3.3; $p<0.001$), adding information that the predictive value of KIM-1 is not independent of albuminuria.

NAG is a lysosymal enzyme predominantly produced in proximal tubules. It has a high molecular weight of 130-140 kDa and therefore not subject to glomerular filtration. It is a sensitive marker of tubular impairment. Elevated urinary NAG is also associated with acute graft rejection and tubular necrosis in the early post-transplantation period,[27] as well as with ischemic renal damage.[28] As yet only 1 study investigated the predictive value of NAG for long term outcome in renal transplant recipients. This study, in only 33 patients, paradoxically found that a low urinary NAG concentration is associated with chronic rejection and ultimately graft loss.[29] In contrast, in our much larger cohort we find that urinary NAG at baseline is significantly and positively associated with graft failure, also after correction for age, sex and eGFR at baseline. However, after adjustment for albuminuria NAG does not significantly predict graft failure anymore.

H-FABP is an intracellular carrier protein and has a low molecular mass of 15 kDa. It is present in the cytoplasm of human distal tubular cells. Little is known about urinary H-FABP in human renal disease. It predicts prognosis in patients with idiopathic membranous nephropathy.[30] We found that urinary H-FABP predicts graft failure in the crude model, suggesting that distal tubuli are also affected in chronic allograft nephropathy.

NGAL is a 25 kDa protein bound to gelatinase from neutrophils and normally expressed at very low levels in human tissues, including the kidney. Expression is augmented in injured epithelia. [31] NGAL has been investigated extensively in acute kidney disease and the acute phase after transplantation, and more recently in ischemic renal function impairment in

heart failure.[32] Little is known about NGAL in the chronic phase after kidney transplantation. One study in patients with a stable graft showed that subclinical tubulitis was associated with higher levels of NGAL.

The finding that albuminuria outperforms proteinuria is in line with the study of Halimi[11], that also showed that albuminuria is a good predictor of graft loss, even in non-proteinuric patients. This cohort has differences compared to our study. Mean donor age is lower in our study (36.9 vs 48 years), partly because our percentage of deceased donors is lower (86 vs 99%), and our follow-up time for graft failure is longer (median follow-up 4.7 vs 3.33 years). Furthermore, we not only looked at albuminuria and proteinuria, but also at specific tubular damage markers. Thus, although the risk profile for graft failure might be lower in our cohort, we show that albuminuria predicts graft failure better than proteinuria.
With respect to change in eGFR during follow-up, there are to our knowledge no other studies in kidney transplant recipients investigating the predictive value of albuminuria, proteinuria and tubular damage markers. Our data indicate that albuminuria predicts change in eGFR during follow-up better than proteinuria or tubular damage markers. Furthermore, albuminuria predicts renal function decline independent of age, sex, eGFR at baseline, as do 2 out of 4 tubular damage markers; KIM-1 and H-FABP. In addition, we found that H-FABP is the only biomarker that predicts renal function decline independent of albuminuria. Whereas KIM-1 is a marker of damage of the proximal tubule, H-FABP is a marker of distal tubular damage. These findings thus suggest that not only the proximal tubule, but also the distal tubule is affected in chronic renal allograft injury.

Why does albuminuria predict renal transplant outcome better than proteinuria? In our cohort almost all patients (97%) have proteinuria >0.1g/24hour whereas only 57% of all patients have albuminuria >30 mg/24 hour. This is in line with a study that showed in renal transplant recipients that low-grade proteinuria (<1 g/d) consists mostly of non-albumin-proteins, whereas

massive proteinuria (>1 g/d) consists mostly of albumin.[33] Total protein excretion in our patients will consist of a large variety in proteins, with very different charge and size characteristics. The more uniform character of albuminuria could make albuminuria outperform proteinuria in predicting kidney outcome.

We found that all tubular damage markers predict graft failure in crude models. However, when albuminuria is added, these markers do not have additive predictive value. The only exception is H-FABP, but this tubular damage marker had additive predictive value for only change in eGFR.. These data suggest that albuminuria in these patients reflects or inflicts tubular damage. Furthermore, this observation makes that the clinical use of these tubular biomarkers on top of albuminuria to predict renal outcome in stable renal transplant recipients is limited.

We acknowledge that this study has limitations. First, it is a single-center study and the predictive capacity of the investigated biomarkers needs to be confirmed in multicenter studies. Second, the renal transplant recipients were included at different time points after transplantation. This could theoretically induce healthy survivor bias. It is therefore advisable to investigate the predictive capacity of the urinary biomarkers on renal outcome at a fixed time-point after transplantation. Patients were included at least one year after transplantation and 80% had proteinuria <1 gram/day. The validity of the results is therefore limited to such selected population. Furthermore, the investigated biomarkers should be analyzed in relation to the histological diagnosis. Strengths of this study are that, as far as we know, it is the first study to investigate the predictive value of these tubular damage markers on renal transplant outcome in stable renal transplant recipients. We not only looked at graft failure but also at renal function decline, which may enable much earlier identification of patients at risk. Furthermore, we studied several tubular damage markers representing different functional entities of the tubule. Lastly, this study has been performed in a relatively large number of subjects and has sufficient duration of follow up.

In conclusion, we show that albuminuria outperforms proteinuria in predicting graft failure and renal function decline. We also show that all investigated tubular damage markers predict graft failure in a crude model. However, when adjusted for age, sex and baseline eGFR only KIM-1 predicts both change in eGFR and graft failure during follow-up. When albuminuria is entered into the models no tubular damage marker has added value in predicting both renal outcome measures. Therefore, we conclude that assessing tubular damage markers has limited clinical value in renal transplant recipients. In contrast, albuminuria is a strong predictor of both eGFR loss and graft failure, independent of all four tubular damage markers and proteinuria. Therefore, this study indicates that albuminuria instead of proteinuria, may be superior for identification of subjects at risk for renal allograft failure who may benefit from intervention or change in therapy.

Disclosure

None of the authors has anything to declare.

Acknowledgment

We thank A.J. Meesters for statistical assistance. This study was supported by grants from the Dutch Kidney Foundation (Nierstichting Nederland C00.1877) and from the Netherlands Organisation for Scientific Research (NWO-AGIKO 920-03-181)

Reference List

1. Hariharan S, Johnson CP, Bresnahan BA, Taranto SE, McIntosh MJ, Stablein D. Improved graft survival after renal transplantation in the United States, 1988 to 1996. *N Engl J Med* 2000; 342: 605-612

2. Kreis HA, Ponticelli C. Causes of late renal allograft loss: chronic allograft dysfunction, death, and other factors. *Transplantation* 2001; 71: SS5-SS9

3. Joosten SA, Sijpkens YW, van Kooten C, Paul LC. Chronic renal allograft rejection: pathophysiologic considerations. *Kidney Int* 2005; 68: 1-13

4. Massy ZA, Guijarro C, Wiederkehr MR, Ma JZ, Kasiske BL. Chronic renal allograft rejection: immunologic and nonimmunologic risk factors. *Kidney Int* 1996; 49: 518-524

5. Chapman JR, O'Connell PJ, Nankivell BJ. Chronic renal allograft dysfunction. *J Am Soc Nephrol* 2005; 16: 3015-3026

6. Racusen LC. The Banff schema and differential diagnosis of allograft dysfunction. *Transplant Proc* 2004; 36: 753-754

7. Marsden PA. Predicting outcomes after renal transplantation--new tools and old tools. *N Engl J Med* 2003; 349: 182-184

8. Lachenbruch PA, Rosenberg AS, Bonvini E, Cavaille-Coll MW, Colvin RB. Biomarkers and surrogate endpoints in renal transplantation: present status and considerations for clinical trial design. *Am J Transplant* 2004; 4: 451-457

9. Kaplan B, Schold J, Meier-Kriesche HU. Poor predictive value of serum creatinine for renal allograft loss. *Am J Transplant* 2003; 3: 1560-1565

10. Kasiske BL, Andany MA, Danielson B. A thirty percent chronic decline in inverse serum creatinine is an excellent predictor of late renal allograft failure. *Am J Kidney Dis* 2002; 39: 762-768

11. Halimi JM, Buchler M, Al Najjar A *et al*. Urinary albumin excretion and the risk of graft loss and death in proteinuric and non-proteinuric renal transplant recipients. *Am J Transplant* 2007; 7: 618-625

12. Pelsers MM. Fatty acid-binding protein as marker for renal injury. *Scand J Clin Lab Invest Suppl* 2008; 241: 73-77

13. van Ree RM, de Vries AP, Oterdoom LH *et al*. Abdominal obesity and smoking are important determinants of C-reactive protein in renal transplant recipients. *Nephrol Dial Transplant* 2005; 20: 2524-2531

14. Kasiske BL, Heim-Duthoy KL, Tortorice KL, Rao KV. The variable nature of chronic declines in renal allograft function. *Transplantation* 1991; 51: 330-334

15. Bosma RJ, Doorenbos CR, Stegeman CA, van der Heide JJ, Navis G. Predictive performance of renal function equations in renal transplant recipients: an analysis of patient factors in bias. *Am J Transplant* 2005; 5: 2193-2203

16. van Ree RM, Oterdoom LH, de Vries AP *et al*. Circulating markers of endothelial dysfunction interact with proteinuria in predicting mortality in renal transplant recipients. *Transplantation* 2008; 86: 1713-1719

17. Wagener G, Jan M, Kim M *et al*. Association between increases in urinary neutrophil gelatinase-associated lipocalin and acute renal dysfunction after adult cardiac surgery. *Anesthesiology* 2006; 105: 485-491

18. Parikh CR, Jani A, Mishra J *et al*. Urine NGAL and IL-18 are predictive biomarkers for delayed graft function following kidney transplantation. *Am J Transplant* 2006; 6: 1639-1645

19. Ronco C. N-GAL: diagnosing AKI as soon as possible. *Crit Care* 2007; 11: 173

20. Ichimura T, Hung CC, Yang SA, Stevens JL, Bonventre JV. Kidney injury molecule-1: a tissue and urinary biomarker for nephrotoxicant-induced renal injury. *Am J Physiol Renal Physiol* 2004; 286: F552-F563

21. van Timmeren MM, Bakker SJ, Vaidya VS *et al*. Tubular kidney injury molecule-1 in protein-overload nephropathy. *Am J Physiol Renal Physiol* 2006; 291: F456-F464

22. Waanders F, van Timmeren MM, Stegeman CA, Bakker SJ, van Goor H. Kidney injury molecule-1 in renal disease. *J Pathol* 2010; 220: 7-16

23. Kramer AB, van Timmeren MM, Schuurs TA *et al*. Reduction of proteinuria in adriamycin-induced nephropathy is associated with reduction of renal kidney injury molecule (Kim-1) over time. *Am J Physiol Renal Physiol* 2009; 296: F1136-F1145

24. Waanders F, Vaidya VS, van Goor H *et al*. Effect of renin-angiotensin-aldosterone system inhibition, dietary sodium restriction, and/or diuretics on urinary kidney injury molecule 1 excretion in nondiabetic proteinuric kidney disease: a post hoc analysis of a randomized controlled trial. *Am J Kidney Dis* 2009; 53: 16-25

25. van Timmeren MM, van den Heuvel MC, Bailly V, Bakker SJ, van GH, Stegeman CA. Tubular kidney injury molecule-1 (KIM-1) in human renal disease. *J Pathol* 2007; 212: 209-217

26. van Timmeren MM, Vaidya VS, van Ree RM *et al*. High urinary excretion of kidney injury molecule-1 is an independent predictor of graft loss in renal transplant recipients. *Transplantation* 2007; 84: 1625-1630

27. Kuzniar J, Marchewka Z, Krasnowski R, Boratynska M, Dlugosz A, Klinger M. Enzymuria and low molecular weight protein excretion as the differentiating marker of complications in the early post kidney transplantation period. *Int Urol Nephrol* 2006; 38: 753-758

28. Loef BG, Henning RH, Epema AH *et al*. Effect of dexamethasone on perioperative renal function impairment during cardiac surgery with cardiopulmonary bypass. *Br J Anaesth* 2004; 93: 793-798

29. Kotanko P, Margreiter R, Pfaller W. Reduced renal allograft survival is related to low urinary N-acetyl-beta-D-glucosaminidase excretion during the first posttransplant month. *Transplantation* 1996; 61: 388-392

30. Hofstra JM, Deegens JK, Steenbergen EJ, Wetzels JF. Urinary excretion of fatty acid-binding proteins in idiopathic membranous nephropathy. *Nephrol Dial Transplant* 2008;

31. Mishra J, Ma Q, Prada A *et al*. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *J Am Soc Nephrol* 2003; 14: 2534-2543

32. Damman K, van Veldhuisen DJ, Navis G, Voors AA, Hillege HL. Urinary neutrophil gelatinase associated lipocalin (NGAL), a marker of tubular damage, is increased in patients with chronic heart failure. *Eur J Heart Fail* 2008; 10: 997-1000

33. Halimi JM, Matthias B, Al Najjar A *et al*. Respective predictive role of urinary albumin excretion and nonalbumin proteinuria on graft loss and death in renal transplant recipients. *Am J Transplant* 2007; 7: 2775-2781

Supplementary tables

Supplementary table 1 *Correlation between all 24 hour damage marker excretions.*

	Protein	*Albumin*	*KIM-1*	*NGAL*	*NAG*	*H-FABP*
Protein		0.46 *	0.35 *	0.33 *	0.34 *	0.24 *
Albumin	0.46 *		0.54 *	0.18 *	0.36 *	0.27 *
KIM-1	0.35 *	0.54 *		0.10 **	0.39 *	0.26 *
NGAL	0.33 *	0.18 *	0.10 **		0.24 *	0.18 *
NAG	0.34 *	0.36 *	0.39 *	0.24 *		0.27 *
H-FABP	0.24 *	0.27 *	0.26 *	0.18 *	0.27 *	

Data are given as Spearmans Rho. * Correlation is significant at the 0.001 level (2-tailed). ** Correlation is significant at the 0.05 level (2-tailed).

Sensitivity analysis by using biomarker/creatinine ratio instead of 24h biomarker excretion

Supplementary table 2 Association between biomarkers at baseline and death censored graft failure during follow-up

	Model 1		*Model 2*		*Model 3*			
	Biomarker (HR)	p-value	Biomarker (HR)	p-value	Biomarker (HR)	p-value	Albuminuria (HR)	p-value
- Proteinuria	2.1 (1.5-2.9)	<0.001	1.7 (1.3-2.4)	0.001	1.2 (0.8-1.8)	0.3	1.8 (1.2-2.8)	0.007
- Albuminuria	3.4 (2.4-4.9)	<0.001	2.1 (1.5-3.0)	<0.001	NA	NA	NA	NA
Proximal tubular								
- KIM-1	3.7 (20.-6.7)	<0.001	2.2 (1.2-3.8)	0.007	1.2 (0.6-2.3)	0.6	20. (1.3-3.0)	0.002
- NAG	4.2 (2.6-6.9)	<0.001	2.0 (1.2-3.3)	0.006	1.6 (0.9-2.7)	0.09	1.9 (1.3-2.8)	<0.001
- NGAL	1.7 (1.3-2.2)	<0.001	1.4 (1.0-1.8)	0.03	1.2 (0.9-1.6)	0.2	2.0 (1.4-2.9)	<0.001
Distal tubular								
- H-FABP	1.8 (1.3-2.4)	<0.001	1.3 (0.9-1.7)	0.1	1.1 (0.8-1.5)	0.6	2.1 (1.4-3.0)	<0.001

	Model 4			
	Biomarker (HR)	p-value	Albuminuria (HR)	p-value
- Proteinuria	1.3 (0.8-2.0)	0.3	1.8 (1.1-3.0)	0.02
- Albuminuria	NA	NA	2.1 (1.4-3.2)	<0.001
Proximal tubular				
- KIM-1	1.1 (0.6-2.2)	0.7	2.0 (1.2-3.3)	0.007
- NAG	1.6 (0.9-2.8)	0.09	2.0 (1.4-2.9)	0.001
- NGAL	1.3 (1.0-1.7)		2.0 (1.4-3.0)	<0.001
Distal tubular				
- H-FABP	1.1 (0.7-1.5)	0.8	2.1 (1.4-3.2)	0.001

Data are given as Hazard Rates (95% Confidence Interval) and p-values. Biomarker excretions are 2log transformed, resulting in expression of Hazard Ratios indicating a risk for death censored graft failure per two fold increase of the biomarker excretion under investigation. 2log biomarker excretions have been converted to a Z-scale, enabling Hazard Rates to be compared with another. Model 1: Crude model ; model 2: Adjustment for age, sex and eGFR; model 3: Model 2 + albuminuria; model 4: Model 3 + time between renal Tx and baseline, donor age, living related or cadaveric Tx, BMI, MAP, smoking, total cholesterol, duration warm ischemia time, duration cold ischemia time, acute rejections, use of calcineurin inhibitors, diabetes mellitus and daily dose of prednisolon at baseline.

Sensitivity analysis by using biomarker/creatinine ratio instead of 24h biomarker excretion.

Supplementary table 3 Association between biomarkers at baseline and change in eGFR per year during follow-up

	Model 1		*Model 2*		*Model 3*			
	Biomarker (Std.β)	p-value	Biomarker (Std.β)	p-value	Biomarker (Std.β)	p-value	Albuminuria (Std.β)	p-value
- Proteinuria	-0.13 (-0.44; 0.18)	0.4	-0.22 (-0.54; 0.10)	0.2	-0.13 (-0.24; 0.49)	0.5	-0.72 (-1.1; -0.34)	<0.001
- Albuminuria	-0.57 (-0.88; -0.25)	<0.001	-0.65 (-0.97; -0.32)	<0.001	NA	NA	NA	NA
Proximal tubular								
- KIM-1	-0.46 (-0.76; 0.16)	0.003	-0.54 (-0.85; -0.24)	<0.001	-0.32 (-0.66; 0.02)	0.07	-0.49 (-0.85; -0.13)	0.008
- NAG	-0.20 (-0.50; -0.11)	0.2	-0.33 (-0.64; -0.01)	0.05	-0.15 (-0.48; 0.17)	0.4	-0.61 (-0.94; 0.27)	<0.001
- NGAL	-0.02 (-0.29; 0.33)	0.9	-0.22 (-0.59; 0.14)	0.2	-0.02 (-0.39; 0.36)	0.9	-0.64 (-0.98; -0.31)	<0.001
Distal tubular								
- H-FABP	-0.43 (-0.73; -0.12)	0.007	-0.51 (-0.82; -0.20)	0.001	-0.36 (-0.68; 0.03)	0.03	-0.56 (-0.89; -0.23)	0.001

	Model 4			
	Biomarker (HR)	p-value	Albuminuria (HR)	p-value
- Proteinuria	0.13 (-0.25; 0.50)	0.51	-0.70 (-1.10; -0.31)	0.001
- Albuminuria	NA	NA	-0.63 (-0.98; -0.29)	<0.001
Proximal tubular				
- KIM-1	-0.22 (-0.58; 0.13)	0.21	-0.52 (-0.91; -0.13)	0.009
- NAG	-0.12 (-0.46; 0.21)	0.46	-0.60 (-0.95; -0.24)	0.001
- NGAL	-0.05 (-0.43; 0.33)	0.79	-0.62 (-0.97; -0.26)	0.001
Distal tubular				
- H-FABP	-0.32 (-0.65; 0.01)	0.06	-0.55 (-0.91; -0.20)	0.002

Data are given as standardized β and p-values. Biomarker excretions are 2log transformed to fulfil requirements of linear regression analysis. Standardized β show increased risk on 1 ml/ min/per year decrease in eGFR per two fold increase of biomarker excretion. Model 1: Crude model; model 2: Adjustment for age, sex and eGFR; model 3: Model 2 + albuminuria; model 4: Model 3 + time between renal Tx and baseline, donor age, living related or cadaveric Tx, BMI, MAP, smoking, total cholesterol, duration warm ischemia time, duration cold ischemia time, acute rejections, use of calcineurin inhibitors, diabetes mellitus and daily dose of prednisolon at baseline.

Chapter 6

Glomerular and tubular damage markers in subjects with progressive albuminuria: results of a nested case-control study

submitted

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Abstract

Albuminuria is associated with risk for renal and cardiovascular disease. It is difficult to predict which subjects will progress in albuminuria. We investigated whether assessment of urinary markers representing damage to different parts of the nephron help to identify subjects that will progress in albuminuria.

Subjects were selected from a prospective community-based cohort study with serial follow-up (PREVEND, n=8592) and defined as progressor if they were in the 20% with most rapid annual increase in albuminuria and reached albuminuria 150 mg/d during follow-up. Subjects with known renal disease were excluded. Each progressors was matched to 2 control subjects, based on age, sex and baseline albuminuria. IgG and IgG-4 were measured as glomerular markers, KIM-1, NAG, β-2-microglobulin, cystatin C as proximal tubular damage markers, H-FABP as marker of distal tubular damage and NGAL and MCP-1 as inflammatory markers.

After a median follow-up of 9.3 yrs, 183 subjects met criteria for progressive albuminuria. Baseline clinical characteristics were comparable between progressors and controls. However, both urinary and fractional excretion of IgG were significantly higher in progressors ($p<0.001$), whereas urinary and fractional excretion of all proximal tubular markers except cystatin C were lower (all $p<0.01$).

These data suggest that albuminuria associated with glomerular damage is more likely to progress, whereas albuminuria associated with tubulo-interstitial damage is more likely to remain stable.

Introduction

Albuminuria predicts cardiovascular events and renal function decline in patients with diabetes mellitus[1] and hypertension[2], in elderly subjects[3] and even in the general population[4-7]. A spontaneous increase in albuminuria is even more strongly associated with accelerated renal function decline and mortality in patients with diabetes.[8] This has also been demonstrated for cardiovascular events in the general population.[9]

Albuminuria is the result of two counteracting processes; glomerular leakage and tubular reabsorption. It has been argued that albuminuria (especially in the micro-albuminuric range) is a marker of glomerular damage related to generalized atherosclerosis[10]. However, higher levels of albuminuria may also be explained by another process. According to the tubular hypoxia hypothesis[11] generalized atherosclerosis decreases blood flow in peritubular capillaries. This results in impaired oxygen diffusion and oxygen supply to tubular cells, leading to tubulo-interstitial damage and interstitial fibrosis. In turn, these processes lead to impaired tubular albumin reabsorption.

Recently, it has become possible to measure in urine samples markers that represent damage to different parts of the nephron.[12] These damage markers have initially drawn much attention as possible early predictors of acute kidney injury, but lately also as predictors for progressive CKD.[13-16] In these studies progressive CKD was defined as loss of GFR. As yet, no study has investigated whether progressive albuminuria is accompanied by a higher excretion of glomerular or tubular damage markers.

We therefore measured the excretion of various of these markers in urine samples of subjects from the general population that had progressive albuminuria and of matched control subjects. As glomerular damage markers we measured IgG and IgG-4; as proximal tubular damage markers KIM-1, NAG, β-2-microglobulin, cystatin C; as distal tubular damage marker H-FABP and as general inflammation markers NGAL and MCP-1.

Methods

Study population

This study is performed as a nested case-control study using information and samples obtained from subjects participating in the Prevention of Renal and Vascular End Stage Disease (PREVEND) study. The PREVEND study is a prospective cohort study, that investigates the association of albuminuria with renal and cardiovascular disease progression. Details of this study have been published elsewhere.[17] The PREVEND study was approved by the medical ethics committee of our institution and conducted in accordance with the guidelines of the Declaration of Helsinki. All participants gave written informed consent.

Measurements and definitions

At the baseline and follow-up screening rounds, all participants completed a questionnaire on demographics, disease history, smoking habits and use of medication. For each screening round, participants visited an outpatient unit. During this visit, height, weight and blood pressure was measured (Dinamap XL Model 9300; Johnson-Johnson Medical, Tampa, FL), and a fasting blood sample was drawn. Hypertension was defined according the JNC-7 criteria as use of antihypertensive drugs, a systolic blood pressure >140 mmHg, or a diastolic blood pressure >90 mmHg. Diabetes Mellitus was defined according the ADA criteria as use of glucose lowering drugs, a fasting glucose >7 mmol/L or a non-fasting glucose >11.1 mmol/L. Hypercholesterolemie was defined as total cholesterol >= 6.0 mmol/L. Concentrations of cholesterol, glucose, and creatinine were measured using standard methods. Furthermore, plasma samples used for biomarker measurements were stored at -80 ºC. GFR was estimated (eGFR) with the modified Modification of Diet in Renal Disease (MDRD) formula, taking into account gender, age, race, and serum creatinine concentration. At each screening round two 24-h urine samples were collected. Urine samples for biomarker measurements were stored at -20 C. Urinary albumin concentration was determined in fresh urine samples by nephelometry with a

threshold of 2.3 mg/L and intra- and interassay coefficients of variation (CV) of 2.2 and 2.6%, respectively (BNII; Dade Behring Diagnostic, Marburg, Germany). Urinary albumin excretion (UAE) is given as the mean of the two 24-h UAEs.

Definition of cases and controls

For the present analyses subjects were excluded when the were known with renal disease according to their questionnaire or in case baseline albuminuria exceeded 300 mg/24h (n=381), leaving 8,211 subjects. Change in albuminuria during follow-up was assessed as last available albuminuria value during follow-up minus baseline albuminuria, divided by follow-up time in years. Subjects were defined as having progressive albuminuria (*cases*) when they belonged to the 20% of subjects with the most rapid progression in albuminuria, and that had an albuminuria in excess of 150 mg/24h during follow-up. These subjects were 1:2 randomly matched to *controls,* with matching criteria being sex, age and baseline albuminuria.

Measurements of Damage Markers

After thawing urine and plasma samples were vortexed and subsequently centrifuged (14.000 rpm). The supernatant was used for measurements. All urinary as well as all plasma biomarkers were determined in one run. As markers of glomerular damage we measured urinary Immunoglobulin G (IgG) and Immunoglobulin G4 (IgG-4)[18,19] The tubular injury markers investigated here reflect injury to different segments of the nephron and are mediated by different processes. Kidney-Injury-Molecule 1 (KIM-1), N-acetyl-β-D-glucosaminidase (NAG), Cystatine C and ß-2-microglobuline (β2MG) were measured as markers for proximal tubular damage.[20-22] As marker of distal tubular damage we measured heart-type fatty acid-binding protein (H-FABP),[23;24] whereas neutrophil gelatinase-associated lipocalin (NGAL) and monocyte chemoattractant protein-1 (MCP-1) were measured as markers of inflammation.[25;26]

For quantification of IgG and IgG-4, NGAL, β2MG, MCP-1, and H-FABP we used direct sandwich-enzyme-linked immunosorbent assays using monoclonal coating antibodies and labelled polyclonal detection antibodies on a Maxisorp plate (Nunc, Denmark) in which is the concentration of the analyte was determined spectrophotometrically by conversion of *o*-phenylenediamine by Horse-Radish Peroxidase label. H-FABP antibodies were obtained from Hytest (Turku, Finland), whereas KIM-1, β-2-microglobulin, MCP-1 and NGAL antibodies were obtained from R&D systems (Minneapolis, USA). The intra-assay coefficients of variation of these ELISA's were 9.3%, 7.4%, 9.7%,15.7 and 6.8% respectively. Cystatin C was measured by nephelometry (reagents obtained from Siemens, Marburg, Germany, intra-assay CV 2.7%). Urinary concentration of NAG was measured using a modified enzyme assay according to Lockwood and corrected for nonspecific conversion (HaemoScan, Groningen, The Netherlands, intra-assay CV 3.1%). All samples were measured in duplicate in both urine and plasma. Fractional excretions were calculated as: FE damage marker = $([\text{marker}]_u \times [\text{creat}]_p) / ([\text{marker}]_p \times [\text{crea}]_u)) \times 100\%$, where abbreviations are : FE, fractional excretion; crea, creatinine; u, urinary; p, plasma.

Statistical analyses

Analyses were performed with SPSS version 18.0 (SPSS Inc., Chicago, IL). Parametric variables are expressed as mean ± standard deviation (SD), whereas non-parametric variables are given as median (25^{th} and 75^{th} percentile). P-values for differences in damage markers between progressors and controls were tested using a Mann-Whitney test.

Correlations and corresponding p-values are expressed as Spearmans correlation coefficients (rho). Glomerular charge selectivity was calculated by dividing the fractional excretion of the positively charged total IgG by the fractional excretion of the negatively charged IgG-4. For all analyses a two sided $p< 0.05$ was considered to indicate statistical significance.

Results

The selection of subjects with progression in albuminuria ("progressors") from the general PREVEND cohort is shown in figure 1. From a total of 8,394 subjects at baseline, 183 met our criteria for defining progression in albuminuria. Of note, for each subject the last available information during follow-up was used to define progressive albuminuria. For 111 subjects information was available of 4 screening rounds, for 44 subjects information was available of 3 screening rounds, and for 28 subjects information was available of only 2 screening rounds. These subjects were 1:2 matched on age, sex and baseline albuminuria to subjects not selected as progressor. Characteristics at baseline and their last follow-up visit are shown in table 1 for progressors and controls. Progressors were in general male (77%), 59 years old (11) and had a median urinary albumin excretion of 58 mg/24h (33-106). Clinical characteristics between progressors and matched controls were in general similar, although progressors used more antihypertensive drugs (41% vs 27%), but had a lower diastolic blood pressure (79 vs 81 mmHg) and a slightly higher BMI (28 vs 27 kg/m^2). Baseline eGFR and mean follow-up time were comparable between progressors and controls (75 vs 76 ml/min, and 9.4 vs 9.3 years, respectively). During follow-up progressors had an increase in albuminuria from 58 to 254 mg/24h ($p<0.001$), whereas albuminuria decreased slightly in controls (from 52 to 39 mg/24h, $p<0.001$).

Table 1 Characteristics at baseline and last follow-up visit of subjects with progressive albuminuria ("progressors") and their controls (matched for age, sex and baseline albuminuria)

	Cohort, n=8394	***Progressors, n=183***		***Controls, n=366***		
	Baseline	Baseline	Last follow-up visit	Baseline	Last follow-up visit	p-value
Age (yr)	50 (13)	59 (11)	67 (11)	59 (11)	67 (11)	0.88
Male (%)	50	77	77	77	77	0.97
Follow-up (years)	-	-	9.4 (0.8)	-	9.3 (0.8)	-
Smoking (%)	38	40	23	36	18	0.39
Baseline history CVD (%)	5	14	-	11	-	0.24
BMI (kg/m^2)	26.1 (4.2)	28.3 (4.1)	28.7 (4.8)	27.5 (4.1)	27.6 (4.8)	0.03
SBP (mmHg)	129 (20)	141 (21)	146 (23)	145 (23)	138 (20)	0.076
DBP (mmHg)	74 (10)	79 (9)	79 (10)	81 (10.6)	77 (9)	0.024
Blood pressure lowering drugs (%)	15	41	60	27	58	0.001
ACEi/ARB (%)	5.5	14	-	36	-	0.36
Hypertension (%)	33	66	78	65	69	0.78
Cholesterol (mmol/L)	5.6 (1.1)	5.8 (1.2)	4.9 (1.2)	5.9 (1.1)	4.9 (1.1)	0.068
Lipid lowering drugs (%)	6	14	38	11	35	0.42
Hypercholesterolemia (%)	40	47	54	53	50	0.18
Glucose (mmol/L)	4.7 (4.3-5.1)	5.0 (4.6-5.6)	5.5 (4.9-6.5)	5.0 (4.6-5.7)	5.4 (4.8-6.1)	0.35
Glucose lowering drugs (%)	2	4	18	5	17	0.74
Diabetes Mellitus	3	12	21	11	18	0.90
eGFR (ml/min)	81 (14)	75 (17)	74 (24)	76 (14)	77 (19)	0.78
Albuminuria (mg/24h)	9 (6-17)	58 (33-106)	254 (188-410)	52 (31-88)	39 (17-80)	0.24

P-values are shown for difference in baseline values between progressors and controls. Mean (SD) or median (25th -75th percentile). P-values are calculated using Mann Whitney U test. *Abbreviations are:* CVD, cardiovascular disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; ACEi, Angiotensin Converting Enzyme inhibitor; ARB, Angiotensin II Receptor Blockers; eGFR, estimated Glomerular Filtration Rate; UAE, urinary albumin excretion.

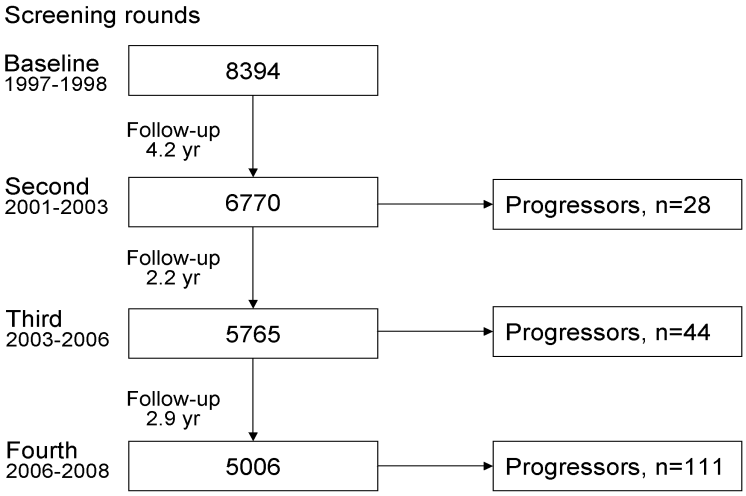

Figure 1 Selection of subjects with progressive albuminuria (n=183) from the PREVEND cohort.

Urinary excretion of renal damage markers

Table 2 Baseline urinary excretions of renal damage markers at baseline

	Progressors	*Controls*	*p-value*
- Albumin (mg/24h)	58 (33-106)	52 (31-88)	0.24
Glomerular			
- IgG (µg/24g)	1285 (926-2181)	1118 (696-2039)	0.009
- IgG-4 (µg/24g)	158 (0-239)	174 (0-290)	0.14
Proximal tubular			
- KIM-1 (µg/24h)	3.9 (1.9-7.0)	7.4 (3.2-15.0)	<0.001
- CysC (µg/24h)	0.011 (0.005-0.030)	0.015 (0.005-0.041)	0.24
- β-2-microglobulin (µg/24h)	103 (39-395)	169 (58-641)	0.004
- NAG (U/24h)	0.011 (0.000-0.255)	0.694 (0.000-1.863)	<0.001
Inflammatory			
- NGAL (µg/24h)	5.5 (2.7-9.6)	6.7 (3.9-11.4)	0.011
- MCP-1 (ng/24h)	467 (261-825)	741 (522-911)	<0.001
Distal tubular			
- H-FABP (µg/24h)	3.4 (0.8-7.2)	2.0 (5.9-7.3)	0.083

Medians (25-75 pct). P-values are calculated using Mann-Whitney test

Table 2 shows the urinary excretion of markers representing damage to different segments of the nephron. As listed, the glomerular marker total IgG was significantly higher in progressors, whereas negatively charged IgG-4 was similar in both groups. All proximal tubular markers, except cystatin C, and the general inflammation markers NGAL and MCP-1 were significantly lower in progressors compared to controls. In contrast, the distal tubular marker H-FABP tended to be higher in the progressors.

Fractional excretions of renal damage markers

To exclude that differences in plasma concentrations of the damage markers may have caused the differences in urinary excretion between progressors and controls, we also measured also plasma concentrations of these damage markers and calculated their fractional excretions. As listed in table 3, these findings corroborate the results observed with respect to urinary excretion: the fractional excretion of the glomerular marker IgG was significantly higher in progressors, whereas the fractional excretions of all proximal tubular markers, except cystatin C, and of general inflammation markers were significantly lower in progressors. The fractional excretion of the distal tubular marker H-FABP, however, was significantly higher in progressors. The index of the fractional excretions[18;19] of total IgG and IgG-4 was 0.28 (0.11-0.73) for progressors and 0.10 (0.03-0.28) for controls ($p<0.001$).

Table 3 Baseline fractional excretions of renal damage markers (expressed as percentage of creatinine excretion).

	Progressors	*Controls*	*p-value*
Glomerular			
- IgG (%)	$8.0*10^{-5}$ ($5.0-15*10^{-5}$)	$5*10^{-5}$ ($2-16*10^{-5}$)	<0.001
- IgG-4 (%)	$1.1*10^{-4}$ ($0-4.0*10^{-4}$)	$1.0*10^{-4}$ ($0-4.2*10^{-4}$)	0.59
Proximal tubular			
- KIM-1 (%)	13.9 (0.9-1713)	1095 (5.2-4368)	<0.001
- CysC (%)	0.01 (0-0.02)	0.01 (0-0.02)	0.58
- β-2-microglobulin (%)	0.04 (0.01-0.20)	0.07 (0.02-0.36)	0.03
- NAG (%)	$1.2*10^{-3}$ ($0-3.1*10^{-2}$)	$55*10^{-3}$ (0-0.16)	<0.001
Inflammatory			
- NGAL (%)	0.04 (0.02-0.07)	0.05 (0.03-0.10)	0.002
- MCP-1 (%)	7.00 (3.58-11.25)	17.46 (11.05-28.54)	<0.001
Distal tubular			
- H-FABP (%)	1.31 (0.23-6.71)	0.69 (0.09-3.86)	0.03

Medians (25-75 pct). P-values are calculated using Mann Whitney U test.

Correlations between markers

Table 4 shows correlations between all markers under investigation. Data are given separately for progressors and controls. As listed, almost all markers were significantly correlated to albuminuria and to each other.

Table 4 Correlations between the 24hr urinary excretions of albumin and the various damage markers.

A	*albumin*	*IgG-total*	*IgG-4*	*KIM-1*	*CysC*	*B2MG*	*NAG*	*NGAL*	*MCP-1*	*H-FABP*
albumin		**0.51 (<0.001)**	**0.42 (<0.001)**	**0.28 (<0.001)**	**0.45 (<0.001)**	**0.46 (<0.001)**	**0.24 (0.002)**	**0.38 (<0.001)**	-0.05 (0.52)	**0.50 (<0.001)**
IgG-total			**0.42 (<0.001)**	**0.26 (<0.001)**	**0.40 (<0.001)**	**0.47 (<0.001)**	-0.04 (0.62)	**0.35 (<0.001)**	0.08 (0.31)	**0.36 (<0.001)**
IgG-4				**0.26 (0.001)**	**0.40 (<0.001)**	**0.45 (<0.001)**	**0.18 (0.02)**	**0.28 (<0.001)**	0.01 (0.87)	**0.36 (<0.001)**
KIM-1					**0.47 (<0.001)**	**0.41 (<0.001)**	0.08 (0.27)	**0.19 (0.009)**	**0.34 (<0.001)**	**0.25 (0.001)**
CysC						**0.69 (<0.001)**	**0.12 (<0.001)**	**0.35 (<0.001)**	0.13 (0.08	**0.51 (<0.001)**
B2MG							0.14 (0.07)	**0.51 (<0.001))**	-0.08 (0.27)	**0.60 (<0.001)**
NAG								0.02 (0.76)	0.02 (0.80)	**0.30 (<0.001)**
NGAL									-0.14 (0.06)	**0.35 (<0.001)**
MCP-1										-0.08 (0.31)

B	*albumin*	*IgG-total*	*IgG-4*	*KIM-1*	*CysC*	*B2MG*	*NAG*	*NGAL*	*MCP-1*	*H-FABP*
albumin		**0.36 (<0.001)**	**0.35 (<0.001)**	**0.30 (<0.001)**	**0.45 (<0.001)**	**0.48 (<0.001)**	**0.14 (0.01)**	**0.26 (<0.001)**	0.07 (0.22)	**0.33 (<0.001)**
IgG-total			**0.39 (<0.001)**	**0.33 (<0.001)**	**0.33 (<0.001)**	**0.47 (<0.001)**	**0.22 (<0.001)**	**0.44 (<0.001)**	0.07 (0.18)	**0.31 (<0.001)**
IgG-4				**0.28 (<0.001)**	**0.37 (<0.001)**	**0.42 (<0.001)**	-0.007 (0.89)	**0.28 (<0.001)**	**0.20 (<0.001)**	**0.31 (<0.001)**
KIM-1					**0.39 (<0.001)**	**0.49 (<0.001)**	**0.11 (0.04)**	**0.20 (0.009)**	**0.34 (<0.001)**	**0.33 (0.001)**
CysC						**0.74 (<0.001)**	**0.18 (0.001)**	**0.24 (<0.001)**	**0.18 (0.001)**	**0.59 (<0.001)**
B2MG							**0.19 (<0.001)**	**0.45 (<0.001)**	**0.15 (0.003)**	**0.54 (<0.001)**
NAG								**0.31 (<0.001)**	0.01 (0.83)	**0.26 (<0.001)**
NGAL									**0.10 (0.05)**	**0.18 (0.001)**
MCP-1										**0.26 (<0.001)**

Correlations are shown as Spearmans rho and corresponding p-value in subjects with progressive albuminuria (n=183, panel A) and controls (n=366, panel B). Bold print indicates statistical significance.

Sensitivity analyses

Several sensitivity analyses were performed. Theoretically participants defined as progressor or controls could have been misclassified because of urine collection errors at baseline. For instance, when progressors missed a portion of their 24hr urine collection, their baseline albuminuria will be falsely low. When their urine collection at the next visit is correct, they may be misclassified as progressor in albuminuria. Concomitantly these subjects will also have low values for renal damage markers at baseline, since these are measured in the same 24hr urine samples, which may lead to bias. However, 24hr urinary volume and creatinine excretion did not differ between progressors and controls at baseline (1644 vs 1573 ml/24h, p=0.10) and (13.2 vs 13.5 mmol/24h, p=0.29). Furthermore, when all damage marker concentrations were expressed per mmol urinary creatinine this yielded similar results as when 24-hour excretions were studied (data not shown).

Furthermore, we investigated whether a difference in prescription rate of albuminuria lowering drugs during follow-up in controls versus progressors may have influenced our results. When all subjects were excluded that started with blood pressure lowering drugs (including ACEi and ARB's) and glucose lowering drugs, 103 progressors and 195 controls remain. Using these strict criteria essentially similar results were obtained.

Discussion

In the present study, we investigated the 24hr urinary excretion of markers representing damage to different parts of the nephron in subjects that progress in albuminuria and in matched controls. We found that in subjects that progress in albuminuria the urinary excretion of the glomerular marker IgG and the distal tubular damage marker H-FABP were higher when compared to controls. In contrast, the urinary excretion of most proximal tubular damage markers and general inflammation markers were lower in progressors. Analysis of fractional excretion data yielded similar results, suggesting that differences in plasma concentrations did not influence our results.

How do these data compare to current literature? Recently, a number of studies have been published investigating whether urinary excretion of these damage markers is associated with progression of chronic kidney disease. For instance, the glomerular damage marker IgG was found to predict the onset of end-stage-renal-disease in CKD patients.[27] Several markers of damage to the proximal tubule have been reported to predict renal function decline in patients with CKD[14,15,28] and renal transplant recipients.[29] Similar findings have been obtained for the urinary excretion of the distal tubular marker H-FABP[30] and the inflammation markers NGAL and MCP-1.[16;31] It is important to note that in all these studies progression of CKD was expressed as decrease in GFR. However, definition and staging of CKD is not only dependent on level of GFR, but also on level of albuminuria.[32] Therefore progression of CKD can also be expressed as a change in albuminuria. Of note, an increase in albuminuria generally is an early sign of CKD progression, whereas a fall in GFR mostly refers to a later phase of CKD progression. Higher levels of albuminuria are associated with an increased risk for all-cause and cardiovascular mortality and with an increased risk for several kidney related outcomes.[33-35] Furthermore, an increase in albuminuria was especially associated with increased mortality and renal function decline.[8] Little is known how glomerular and tubular damage markers relate to progression in albuminuria. Kern at el reported that in patients with diabetes mellitus urinary NAG predicted the occurrence of albuminuria.[36] We are to our knowledge the first to investigate in subjects selected form a population based cohort how a panel of markers representing damage to different parts of the nephron relate to progression of albuminuria.

A priori we hypothesised that all damage markers would be higher, or at least equal, in subjects that progress in albuminuria when compared to subjects with stable albuminuria. We expected to find that markers related to a specific part of the nephron would be especially increased and helpful to identify subjects that will show progressive albuminuria. We indeed found

that in these subjects at baseline the urinary excretion of markers indicating damage to the glomerulus (IgG) was significantly higher. This suggests that especially damage to the glomerulus is associated with progression in albuminuria. Furthermore, since in our study progressors had higher urinary excretion and fractional excretion of IgG, but not of the neutrally charged IgG-4, this suggests that loss of glomerular size selectivity is associated with progressive albuminuria, rather than loss of charge selectivity. This is corroborated by a higher total IgG / IgG-4 index in progressors compared to controls.

To our surprise we found that markers representing damage to the proximal tubule and markers representing inflammation were significantly lower in the subjects with progressive albuminuria than in controls. In retrospect we think that this may be explained by the fact that by design controls were matched to progressors on baseline albuminuria. We choose for this design because a priori it seemed most suited to answer the question whether it is possible to make a distinction between subjects with a given level of albuminuria that will progress or remain stable. As explained in the introduction section albuminuria is the result of two counteracting processes, glomerular filtration and tubular reabsorption. When controls have similar albuminuria when compared to progressor, but less glomerular loss of albuminuria, they should qualitate qua have less tubular reabsorption of albumin. This may explain why we found proximal tubular damage markers to be higher in controls than in cases. This finding, however, does not deny that subjects with progressive albuminuria have also damage to their proximal tubules. At baseline cases as well as controls had more albuminuria than the overall population of our cohort study. We and others have found that albuminuria is positively associated with the urinary excretion of markers indicating damage tot the proximal tubule.[37;38] This is corroborated in the present study, since we found such an association in cases as well as controls (Table 4A and 4B, respectively).

Our study has limitations that need to be mentioned. First, albuminuria is known to show short-term fluctuations that are not due to real progression. For that reason, however, we choose strict criteria to define progression. Consequently, the subjects that met our definition of progressive albuminuria showed an increase in albuminuria from 58 mg/24h at baseline to 254 mg/24h at the last follow-up visit. Such a rise in albuminuria is unlikely to be the result of random fluctuation, but suggests true progression in albuminuria. Second, all markers were measured in urine samples that have been stored at -20º C. It has been suggested that prolonged frozen storage influences urinary biomarker concentration.[39] However, we recently investigated whether these markers can be measured from frozen samples with sufficient reliability. We found for all damage markers that were used in the present study that concentrations measured in urine samples after storage at -20º C yielded significant associations with the concentrations of these markers when measured in fresh urine samples (data submitted). Furthermore, a possible storage effect would affect samples from progressors and controls to the same extent, since they have been stored under exactly the same conditions. Storage effects are therefore unlikely to account for the differences between progressors and controls that we found.

This study also has several strengths. First, we used 24hr urine collections, which is the gold standard for assessing albumin and damage marker excretions. Other studies use spot urines, which induces more variation.[40] Second, in contrast to most studies investigating the value of these markers in CKD, we measured not only one, but a panel of markers, reflecting damage to different parts of the nephron. Third, our nested case-control study design, selecting subjects with a clinical relevant increase in albuminuria from a large cohort of 8592 persons with serial follow-up with respect to albuminuria during more than 9 years, renders a relatively large number of cases resulting in sufficient power to draw conclusions. Fourth, we investigated not only urinary excretion of these damage markers, but we

also corrected for their plasma concentrations by calculating fractional excretions. Importantly, this analysis yielded similar results.

What may be the implications of this study? Our data suggest that subjects with albuminuria that is associated with glomerular damage are more likely to develop progressive albuminuria than subjects with albuminuria that is associated with proximal tubular damage. However, we found considerable overlap in damage marker excretions between progressors and controls. It will therefore difficult to predict which subject will progress in albuminuria using just one marker. Measuring a panel of markers might be more effective. Determining which marker, or combination of markers, is best suited for this goal necessitates complex analyses investigating among others sensitivity, specificity, predictive value and incremental value of these marker combination beyond traditional risk factors. Such analyses are beyond the scope of the present study, which was designed primarily to have a hypothesis generating pathophysiological character. In that respect it is of interest that we showed that measuring the urinary excretions of these markers provided nearly the same results as measuring their fractional excretions. This renders measuring solely urinary excretions of these markers a reliably method to investigate renal damage.
In conclusion, subjects with albuminuria associated with glomerular damage are more likely to develop progressive albuminuria than subjects with albuminuria that is more associated with proximal tubular damage.

Acknowledgements

We thank Jan Roggeveld and Betttine Haandrikman for their technical assistance, and Dade Behring (Marburg, Germany) for supplying equipment (Behring Nephelometer II) and reagents for nephelometric measurement of urinary albumin concentration. The PREVEND study has been made possible by grants of the Dutch Kidney Foundation. The funding for this study had no role in its design, conduct, or analysis or in the decision to submit the study for publication.

Statement of competing financial interests

None of the authors has anything to declare.

Reference List

1. Mogensen CE: Microalbuminuria predicts clinical proteinuria and early mortality in maturity-onset diabetes. *N Engl J Med* 310:356-360, 1984

2. Wachtell K, Ibsen H, Olsen MH, Borch-Johnsen K, Lindholm LH, Mogensen CE, Dahlof B, Devereux RB, Beevers G, de Faire U, Fyhrquist F, Julius S, Kjeldsen SE, Kristianson K, Lederballe-Pedersen O, Nieminen MS, Okin PM, Omvik P, Oparil S, Wedel H, Snapinn SM, Aurup P: Albuminuria and cardiovascular risk in hypertensive patients with left ventricular hypertrophy: the LIFE study. *Ann Intern Med* 139:901-906, 2003

3. Damsgaard EM, Froland A, Jorgensen OD, Mogensen CE: Microalbuminuria as predictor of increased mortality in elderly people. *BMJ* 300:297-300, 1990

4. Hillege HL, Fidler V, Diercks GF, van Gilst WH, de Zeeuw D, van Veldhuisen DJ, Gans RO, Janssen WM, Grobbee DE, de Jong PE: Urinary albumin excretion predicts cardiovascular and noncardiovascular mortality in general population. *Circulation* 106:1777-1782, 2002

5. Matsushita K, van der Velde M, Astor BC, Woodward M, Levey AS, de Jong PE, Coresh J, Gansevoort RT: Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. *Lancet* 375:2073-2081, 2010

6. van der Velde M, Halbesma N, de Charro FT, Bakker SJ, de Zeeuw D, de Jong PE, Gansevoort RT: Screening for albuminuria identifies individuals at increased renal risk. *J Am Soc Nephrol* 20:852-862, 2009

7. Keane WF, Zhang Z, Lyle PA, Cooper ME, de Zeeuw D, Grunfeld JP, Lash JP, McGill JB, Mitch WE, Remuzzi G, Shahinfar S, Snapinn SM, Toto R, Brenner BM: Risk scores for predicting outcomes in patients with type 2 diabetes and nephropathy: the RENAAL study. *Clin J Am Soc Nephrol* 1:761-767, 2006

8. Spoelstra-de Man AM, Brouwer CB, Stehouwer CD, Smulders YM: Rapid progression of albumin excretion is an independent predictor of cardiovascular mortality in patients with type 2 diabetes and microalbuminuria. *Diabetes Care* 24:2097-2101, 2001

9. Brantsma AH, Bakker SJ, de Zeeuw D, de Jong PE, Gansevoort RT: Extended prognostic value of urinary albumin excretion for cardiovascular events. *J Am Soc Nephrol* 19:1785-1791, 2008

10. El Nahas M: Cardio-Kidney-Damage: a unifying concept. *Kidney Int* 78:14-18, 2010

11. Nangaku M: Chronic hypoxia and tubulointerstitial injury: a final common pathway to end-stage renal failure. *J Am Soc Nephrol* 17:17-25, 2006

12. Waanders F, Navis G, van Goor H: Urinary tubular biomarkers of kidney damage: potential value in clinical practice. *Am J Kidney Dis* 55:813-816, 2010

13. van Timmeren MM, Vaidya VS, van Ree RM, Oterdoom LH, de Vries AP, Gans RO, van Goor H, Stegeman CA, Bonventre JV, Bakker SJ: High urinary excretion of kidney injury molecule-1 is an independent predictor of graft loss in renal transplant recipients. *Transplantation* 84:1625-1630, 2007

14. Peters HP, Waanders F, Meijer E, van den Brand J, Steenbergen EJ, van Goor H, Wetzels JF: High urinary excretion of kidney injury molecule-1 is an independent predictor of end-stage renal disease in patients with IgA nephropathy. *Nephrol Dial Transplant* 2011

15. Branten AJ, du Buf-Vereijken PW, Klasen IS, Bosch FH, Feith GW, Hollander DA, Wetzels JF: Urinary excretion of beta2-microglobulin and IgG predict prognosis in idiopathic membranous nephropathy: a validation study. *J Am Soc Nephrol* 16:169-174, 2005

16. Bolignano D, Lacquaniti A, Coppolino G, Donato V, Campo S, Fazio MR, Nicocia G, Buemi M: Neutrophil gelatinase-associated lipocalin (NGAL) and progression of chronic kidney disease. *Clin J Am Soc Nephrol* 4:337-344, 2009

17. Pinto-Sietsma SJ, Janssen WM, Hillege HL, Navis G, de Zeeuw D, de Jong PE: Urinary albumin excretion is associated with renal functional abnormalities in a nondiabetic population. *J Am Soc Nephrol* 11:1882-1888, 2000

18. Nakamura Y, Myers BD: Charge selectivity of proteinuria in diabetic glomerulopathy. *Diabetes* 37:1202-1211, 1988

19. Hemmelder MH, de Zeeuw D, de Jong PE: Measurement of glomerular charge selectivity in non-diabetic renal disease. *Nephrol Dial Transplant* 12 Suppl 2:57-62, 1997

20. Waanders F, van Timmeren MM, Stegeman CA, Bakker SJ, van Goor H: Kidney injury molecule-1 in renal disease. *J Pathol* 220:7-16, 2010

21. Herget-Rosenthal S, Feldkamp T, Volbracht L, Kribben A: Measurement of urinary cystatin C by particle-enhanced nephelometric immunoassay: precision, interferences, stability and reference range. *Ann Clin Biochem* 41:111-118, 2004

22. Cruz DN, Goh CY, Haase-Fielitz A, Ronco C, Haase M: Early biomarkers of renal injury. *Congest Heart Fail* 16 Suppl 1:S25-S31, 2010

23. Pelsers MM: Fatty acid-binding protein as marker for renal injury. *Scand J Clin Lab Invest Suppl* 241:73-77, 2008

24. Maatman RG, Van Kuppevelt TH, Veerkamp JH: Two types of fatty acid-binding protein in human kidney. Isolation, characterization and localization. *Biochem J* 273 (Pt 3): 759-766, 1991

25. Kuwabara T, Mori K, Mukoyama M, Kasahara M, Yokoi H, Saito Y, Yoshioka T, Ogawa Y, Imamaki H, Kusakabe T, Ebihara K, Omata M, Satoh N, Sugawara A, Barasch J, Nakao K: Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons. *Kidney Int* 75:285-294, 2009

26. Fornoni A, Ijaz A, Tejada T, Lenz O: Role of inflammation in diabetic nephropathy. *Curr Diabetes Rev* 4:10-17, 2008

27. Tofik R, Aziz R, Reda A, Rippe B, Bakoush O: The value of IgG-uria in predicting renal failure in idiopathic glomerular diseases. A long-term follow-up study. *Scand J Clin Lab Invest* 71:123-128, 2011

28. Bazzi C, Petrini C, Rizza V, Arrigo G, Napodano P, Paparella M, D'Amico G: Urinary N-acetyl-beta-glucosaminidase excretion is a marker of tubular cell dysfunction and a predictor of outcome in primary glomerulonephritis. *Nephrol Dial Transplant* 17:1890-1896, 2002

29. Nauta FL, Bakker SJ, van Oeveren W, Navis G, van der Heide JJ, van Goor H, de Jong PE, Gansevoort RT: Albuminuria, Proteinuria, and Novel Urine Biomarkers as Predictors of Long-term Allograft Outcomes in Kidney Transplant Recipients. *Am J Kidney Dis* 2011

30. Hofstra JM, Deegens JK, Steenbergen EJ, Wetzels JF: Urinary excretion of fatty acid-binding proteins in idiopathic membranous nephropathy. *Nephrol Dial Transplant* 23:3160-3165, 2008

31. Tam FW, Riser BL, Meeran K, Rambow J, Pusey CD, Frankel AH: Urinary monocyte chemoattractant protein-1 (MCP-1) and connective tissue growth factor (CCN2) as prognostic markers for progression of diabetic nephropathy. *Cytokine* 47:37-42, 2009

32. Levey AS, de Jong PE, Coresh J, Nahas ME, Astor BC, Matsushita K, Gansevoort RT, Kasiske BL, Eckardt KU: The definition, classification and prognosis of chronic kidney disease: a KDIGO Controversies Conference report. *Kidney Int* 2010

33. van der Velde M, Matsushita K, Coresh J, Astor BC, Woodward M, Levey A, de Jong P, Gansevoort RT, van der Velde M, Matsushita K, Coresh J, Astor BC, Woodward M, Levey AS, de Jong PE, Gansevoort RT, Levey A, El Nahas M, Eckardt KU, Kasiske BL, Ninomiya T, Chalmers J, Macmahon S, Tonelli M, Hemmelgarn B, Sacks F, Curhan G, Collins AJ, Li S, Chen SC, Hawaii Cohort KP, Lee BJ, Ishani A, Neaton J, Svendsen K, Mann JF, Yusuf S, Teo KK, Gao P, Nelson RG, Knowler WC, Bilo HJ, Joosten H, Kleefstra N, Groenier KH, Auguste P, Veldhuis K, Wang Y, Camarata L, Thomas B, Manley T: Lower estimated glomerular filtration rate and higher albuminuria are associated with all-cause and cardiovascular mortality. A collaborative meta-analysis of high-risk population cohorts. *Kidney Int* 79:1341-1352, 2011

34. Astor BC, Matsushita K, Gansevoort RT, van der Velde M, Woodward M, Levey AS, Jong PE, Coresh J, Astor BC, Matsushita K, Gansevoort RT, van der Velde M, Woodward M, Levey AS, de Jong PE, Coresh J, El Nahas M, Eckardt KU, Kasiske BL, Wright J, Appel L, Greene T, Levin A, Djurdjev O, Wheeler DC, Landray MJ, Townend JN, Emberson J, Clark LE, Macleod A, Marks A, Ali T, Fluck N, Prescott G, Smith DH, Weinstein JR, Johnson ES, Thorp ML, Wetzels JF, Blankestijn PJ, van Zuilen AD, Menon V, Sarnak M, Beck G, Kronenberg F, Kollerits B, Froissart M, Stengel B, Metzger M, Remuzzi G, Ruggenenti P, Perna A, Heerspink HJ, Brenner B, de Zeeuw D, Rossing P, Parving HH, Auguste P, Veldhuis K, Wang Y, Camarata L, Thomas B, Manley T: Lower estimated glomerular filtration rate and higher albuminuria are associated with mortality and end-stage renal disease. A collaborative meta-analysis of kidney disease population cohorts. *Kidney Int* 79:1331-1340, 2011

35. Gansevoort RT, Matsushita K, van der Velde M, Astor BC, Woodward M, Levey AS, Jong PE, Coresh J, Gansevoort RT, Matsushita K, van der Velde M, Astor BC, Woodward M, Levey AS, de Jong PE, Coresh J, El Nahas M, Eckardt KU, Kasiske BL, Ninomiya T, Chalmers J, Macmahon S, Tonelli M, Hemmelgarn B, Wang Y, Atkins RC, Polkinghorne KR, Chadban SJ, Shankar A, Klein R, Klein BE, Sacks F, Curhan G, Shlipak M, Sarnak MJ, Katz R, Fried LP, Hallan S, Lydersen S, Holmen J, Lee BJ, Ishani A, Neaton J, Svendsen K, Iseki K, Mann JF, Yusuf S, Teo KK, Gao P, Nelson RG, Knowler WC, Auguste P, Veldhuis K, Camarata L, Thomas B, Manley T: Lower estimated GFR and higher albuminuria are associated with adverse kidney outcomes in both general and high-risk populations. A collaborative meta-analysis of general and high-risk population cohorts. *Kidney Int* 2011

36. Kern EF, Erhard P, Sun W, Genuth S, Weiss MF: Early urinary markers of diabetic kidney disease: a nested case-control study from the Diabetes Control and Complications Trial (DCCT). *Am J Kidney Dis* 55:824-834, 2010

37. Nauta FL, Boertien WE, Bakker SJ, van Goor H, van Oeveren W, de Jong PE, Bilo H, Gansevoort RT: Glomerular and Tubular Damage Markers Are Elevated in Patients With Diabetes. *Diabetes Care* 2011

38. Bolignano D, Lacquaniti A, Coppolino G, Donato V, Fazio MR, Nicocia G, Buemi M: Neutrophil gelatinase-associated lipocalin as an early biomarker of nephropathy in diabetic patients. *Kidney Blood Press Res* 32:91-98, 2009

39. Brinkman JW, de Zeeuw D, Duker JJ, Gansevoort RT, Kema IP, Hillege HL, de Jong PE, Bakker SJ: Falsely low urinary albumin concentrations after prolonged frozen storage of urine samples. *Clin Chem* 51:2181-2183, 2005

40. Witte EC, Lambers Heerspink HJ, de Zeeuw D, Bakker SJ, de Jong PE, Gansevoort R: First morning voids are more reliable than spot urine samples to assess microalbuminuria. *J Am Soc Nephrol* 20:436-443, 2009

Chapter 7

Effect of intensified proteinuria reduction by dual RAAS-blockade and dietary sodium restriction on markers of tubulo-interstitial injury in non-diabetic nephropathy

Submitted

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Abstract

Amelioration of proteinuria-driven tubulo-interstitial injury by renin-angiotensin-aldosterone system (RAAS) blockade and sodium reduction induces renoprotection, and can be monitored by tubular injury markers. We tested whether intensive proteinuria reduction -that is below 0.3 g/day- by sodium restriction and dual RAAS blockade, further decreases a broad panel of tubular markers. Here fore we tested in a cross-over randomized controlled trial 52 non-diabetic renal patients with proteinuria (1.9 [0.9-3.4] g/day) and mildly impaired renal function (69 [50-110] mL/min), and 52 healthy subjects.

Patients were treated with combinations of ACE inhibition (lisinopril 40 mg/d), placebo, angiotensin receptor blockade (valsartan 320 mg/d), regular sodium diet (189±8 mmol Na^+/d), and low sodium diet (106±7 mmol Na^+/d, $p<0.001$): during four randomly-ordered six-week study periods: 1. Lisinopril+placebo +regular sodium diet (baseline), 2. Lisinopril+valsartan+regular sodium diet, 3. Lisinopril+placebo+low sodium diet, 4. Lisinopril+valsartan+low sodium diet. We measured 24-hour urinary excretion of markers of proximal (NAG, KIM-1, β2MG) and distal (H-FABP) tubular injury and inflammation (MCP-1, NGAL).

All tubular injury markers were elevated in the renal patients at baseline. NAG, β2MG, KIM-1 and H-FABP correlated positively with proteinuria, and were reduced along with further proteinuria reduction by combinations of lisinopril, valsartan and low sodium diet. The lowest levels of NAG, β2MG, and H-FAPB were achieved when proteinuria fell below 0.3 g/day. In contrast, MCP-1 and NGAL did not correlate with proteinuria, and were not reduced during proteinuria reduction.

In conclusion, markers of proximal and distal tubular injury and inflammation are elevated in proteinuric renal patients on ACE inhibition, consistent with ongoing renal injury. Intensified treatment with dietary sodium restriction and dual RAAS blockade reduces tubular injury markers in proportion to proteinuria, without improvement of tubular inflammation markers.

Introduction

Reduction of proteinuria and hypertension are the main treatment targets for renoprotection[1;2]. This can be achieved by blockade of the renin-angiotensin-aldosterone system (RAAS) with angiotensin converting enzyme inhibition (ACEi) or angiotensin receptor blockade (ARB) and sodium depletion with dietary sodium restriction or diuretics[3-6]. One of the mechanisms allegedly contributing to the renoprotective effect of proteinuria reduction is amelioration of proteinuria-driven tubulo-interstitial injury[7]. Tubulo-interstitial injury is a main determinant of renal outcome but cannot be assessed directly on a routine basis, as this requires renal biopsy[8;9]. Urinary tubular injury markers might provide a useful non-invasive alternative, as these markers correlate with tubulo-interstitial injury and predict renal outcome[10;11].

It has recently been reported that reduction of proteinuria by combinations of ARB, dietary sodium restriction, and diuretics, is associated with reduction of the tubular injury markers N-Acetyl-β-glucosaminidase (NAG) and Kidney Injury Molecule 1 (KIM-1) in renal patients[12]. Interestingly, the lowest levels of NAG and KIM-1 were achieved when proteinuria fell below the current treatment target of <1.0 g/day, although even in this condition NAG en KIM-1 remained substantially elevated compared to healthy subjects.[13] This may reflect ongoing renal damage and is in line with the hypothesis that further reduction of proteinuria, i.e. to below 0.3 g/day, may augment renoprotection[6]. In the current study, we investigated therefore whether intensified proteinuria reduction to levels below 0.3 g/day by combinations of ACEi, ARB, and dietary sodium restriction, results in further reduction of a broad panel of urinary markers reflecting diverse aspects of tubular injury, in patients with chronic kidney disease (CKD).

Methods

Patients

This is a post-hoc analysis of a randomized double-blind placebo-controlled cross-over multicenter trial. The protocol was described in detail elsewhere[14]. In short, we studied 52 patients with non-diabetic nephropathy. Inclusion criteria were blood pressure ≥ 125/75 mmHg in combination with residual proteinuria ≥ 1.0 g/d during ACEi on maximal dose (lisinopril 40 mg/d), creatinine clearance ≥ 30 mL/min, and age ≥18 years. Exclusion criteria were systolic blood pressure ≥ 180 mmHg, diastolic blood pressure ≥ 110 mmHg, diabetes mellitus, renovascular hypertension, instable renal disease as indicated by a decrease in creatinine clearance ≥ 6 mL/min in the previous year, a cardiovascular event in the previous six months, immunosuppressive treatment, regular use (>1 day/week) of non-steroidal anti-inflammatory drugs, pregnancy or breast feeding.

Protocol

During a run-in period of at least six weeks, patients received ACEi at maximal dose (lisinopril 40 mg/day) and stopped other RAAS blockers. Additional antihypertensives were allowed and kept stable during the study. No dietary intervention took place during the run-in period. After the run-in period patients were treated with combinations of lisinopril 40 mg/d, placebo (PLA), ARB at maximal dose (valsartan 320 mg/d), regular sodium diet (RS; target 200 mmol Na^+/day), and low sodium diet (LS; target intake 50 mmol Na^+/day), during four randomly-ordered six-week study periods: 1. ACEi +PLA+RS, 2. ACEi+ARB+RS, 3. ACEi+PLA+LS, 4. ACEi+ARB+LS. (Figure 1) The drug interventions were double blind, whereas the dietary interventions were open label.

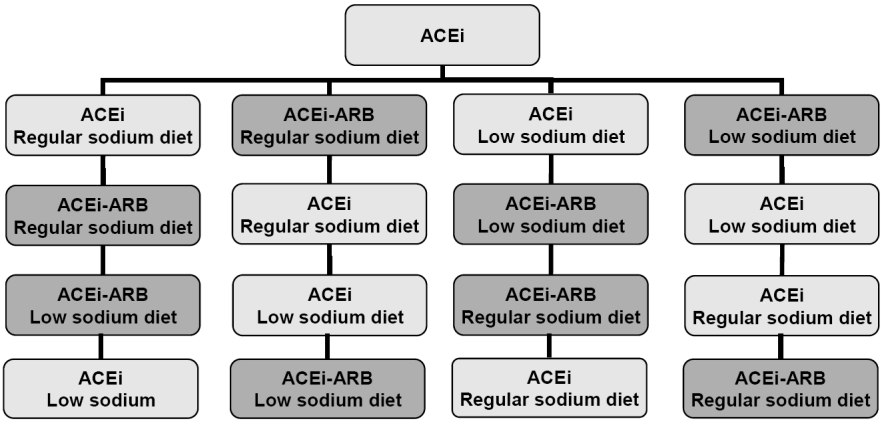


Figure 1 Study design. During a run-in period of ≥6-weeks, ACEi on maximal dose (lisinopril 40 mg/d) was instituted and other RAAS blockers were stopped. Subsequently, patients were treated during four 6-week periods with ACEi on maximal dose (lisinopril 40 mg/d) plus placebo, and ACEi plus ARB on maximal dose (lisinopril 40 mg/d plus valsartan 320 mg/d), combined with consecutively a regular sodium diet and a low sodium diet, in random order.

Healthy controls

Fifty-two race, age and gender matched healthy subjects that had no renal disease (eGFR >60ml/min and albuminuria <30 mg/24h) or diabetes served as controls. These subjects followed a regular sodium diet.

Measurements and calculations

At the end of each 6-week treatment period, patients collected 24 hour urine samples, and blood pressure was measured and blood was sampled after an overnight fast. Proteinuria was measured in 24 hour urine samples with a turbidimetric assay using benzethonium chloride (Modular, Roche Diagnostics, Mannheim, Germany). Blood pressure was measured for 15 minutes at one minute intervals with an automatic device (Dinamap, G E Medical Systems, Milwaukee, WI, USA) in the supine position and we used the mean of the last three readings. Blood electrolytes, proteins, and urinary electrolytes were determined by using an automated multianalyser (Modular, Roche Diagnostics, Mannheim, Germany). We assessed dietary sodium

intake from urinary sodium excretion. Creatinine clearance was calculated from creatinine concentrations in plasma and in 24 hour urine samples.

Damage markers

NAG (N-Acetyl-β-glucosaminidase; 135 kDa) is a lysosymal enzyme that is predominantly produced in the proximal tubule, and released into urine upon cellular damage. Elevated urinary NAG predicts the subsequent occurrence of albuminuria in diabetic patients[16], and was found to predict CKD progression better than proteinuria in non-diabetic CKD[17]. KIM-1 (kidney injury molecule-1; 104 kDa) is a transmembrane glycoprotein that is abundantly expressed on proximal tubular cells and shed into urine, during acute or chronic renal injury.[18-20] No other organs express KIM-1 to a degree that would influence renal excretion of KIM-1.[21] Urinary KIM-1 predicts long-term renal outcome in acute renal injury and renal transplant recipients.[22-24] So far, long-term data on the prognostic significance of urinary KIM-1 in CKD are lacking. ß2MG (β2-microglobulin; 12 kDa) is a component of MHC class 1 molecules, which are present on all nucleated cells. ß2MG is freely filtered through the glomerulus and subsequently reabsorbed by proximal tubular cells. Urinary ß2MG is a marker of proximal tubular reabsorption incapacity and predicts the rate of CKD progression.[25-27] H-FABP (heart-type fatty acid-binding protein; 15kDa) is an intracellular carrier protein present in cytoplasm of distal tubular cells.[28;29] Urinary H-FABP results from release by structurally damaged tubular cells. Elevated urinary H-FABP predicts prognosis in CKD.[30] NGAL (neutrophil gelatinase-associated lipocalin; 25 kDa) is expressed by neutrophils and other epithelial cells. NGAL was found to reflect damage to glomeruli, and proximal and distal tubules.[31-33] Elevated urinary NGAL predicts CKD progression.[34] MCP-1 (monocyte chemoattractant protein-1; 13-30 kDa) is expressed by inflammatory cells such as monocytes, and also by resident renal cells, i.e. mesangial, endothelial, and tubular epithelial cells.[35] Renal cells produce MCP-1 in response to a variety of pro-inflammatory stimuli.[36] Elevated urinary MCP-1 predicts the rate of renal function loss in CKD.[37;38]

We stored (-80˚C) aliquots from 24 hour urine until biomarker analysis. All urine samples were vortexed and centrifuged (14.000 rpm) after thawing. The supernatant was used for measurements. Samples were diluted to obtain the optimal concentration for measurement. All tubular markers were determined in one run. We measured urinary albumin levels by nephelometry (Dade Behring Nephelometer, intra-assay CV 2.7%). For quantification of neutrophil gelatinase-associated lipocalin (NGAL), β2-microglobulin (β2MG), monocyte chemoattractant protein-1 (MCP-1), and heart-type fatty acid-binding protein (H-FABP) we used direct sandwich-enzyme-linked immunosorbent assays using monoclonal coating antibodies and labeled polyclonal detection antibodies on a Maxisorp plate (Nunc, Denmark) in which is the concentration of the analyte was determined spectrophotometrically by conversion of *o*-phenylenediamine by Horse-Radish Peroxidase label. H-FABP, NGAL, β2MG, and MCP-1 antibodies were obtained from Hytest (Turku, Finland, intra-assay CV 9.3%) and R&D systems (Minneapolis, USA, intra-assay CV 6.8 %, 9.7 and 15.7 &, respectively). We measured KIM-1 using microbead based ELISA (microsphere-based Luminex xMAP technology (Luminex, Austin, TX), with polyclonal antibodies raised against the human KIM-1 ectodomain as described previously.[15] The intra-assay variability was less than 15%. Urinary concentration of N-acetyl-β-D-glucosaminidase (NAG) were measured using a modified enzyme assay according to Lockwood and corrected for nonspecific conversion (HaemoScan, Groningen, The Netherlands, intra-assay CV 3.1%).

Statistical analysis

Data are given as mean with standard error (SE) when normally distributed or otherwise as median with interquartile range (IQR). We used paired *t* tests, Wilcoxon signed rank tests, and Pearson's χ^2 tests (which account for the same patients providing data for both treatments) to determine effects of treatment. To determine differences between patients and healthy subjects independent *t* tests or Mann-Whitney U tests were used. Multivariate models

were used to investigate which factors predict change in tubular injury marker excretion. To this purpose, we calculated the change in tubular injury marker excretion from baseline (ACEi+RS) for each treatment period and used this as a dependant variable in the model. Change in proteinuria, change in blood pressure, salt intake and addition of ARB were added as covariates in the model. Alpha was set at $P<0.05$. For all analyses SPSS 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA) was used.

Results

Participants characteristics

CKD patients and healthy controls were matched for age (51 (2) vs. 53 (2) years, p=0.49), gender (83% vs. 73% male, p=0.24) and race (all Caucasian). During ACEi combined with regular sodium diet (ACEi+RS), which was taken as the reference baseline period, CKD patients had overt proteinuria (1.9 (0.9-3.4) g/d), high-normal blood pressure (SBP 134 (3) mmHg, DBP 80 (2) mmHg), and mildly impaired renal function (creatinine clearance 69 (50-110) mL/min). As expected, healthy controls had no relevant albuminuria (8 (6-13) mg/24h, $p<0.001$), normal renal function (creatinine clearance 130 (6) mL/min, $p<0.001$), a lower blood pressure (SBP 122 (2) mmHg, p=0.002; DBP 74 (1) mmHg, p=0.008) compared to CKD patients. Dietary sodium intake, as reflected by urinary sodium excretion, was comparable in CKD patients during ACEi+RS and in controls (189 (8) vs. 198 (12) mmol Na^+/d, p=0.51). Other patient characteristics are shown in Table 1.

Table 1 Patients characteristics

Number of patients	52
Renal diagnosis	
- IgA nephropathy n (%)	15 (29)
- FSGS n (%)	16 (31)
- Membranous NP n (%)	7 (13)
- Hypertensive NP n (%)	6 (12)
- Other / inconclusive n (%)	8 (15)
Use of non-study medication	
- Betablocker n (%)	12 (23)
- Calcium channel blocker n (%)	10 (19)
- Alpha blocker n (%)	6 (12)
- Thiazide diuretic n (%)	8 (15)
- Loop diuretic n (%)	5 (10)
- Lipid lowering agent n (%)	30 (58)

Renal diagnoses and non-study medication as used at the end of the run-in period. Non-study medication was kept stable during the study. Abbreviations: NP, nephropathy; FSGS, focal segmental glomerulosclerosis.

Clinical parameters during the four treatment regimens

In CKD patients, urinary creatinine excretion was comparable during all treatment periods, indicating accurate 24-hour urine collection (Table 2). Dietary sodium intake, as reflected by urinary sodium excretion, was considerably and consistently lower during the LS periods compared with the RS, thus reflecting dietary compliance. Addition of ARB to ACEi resulted in a modest decrease in proteinuria, but LS reduced proteinuria more effectively, and the lowest proteinuria was achieved by combined ARB and LS added to ACEi. ARB did not decrease systolic blood pressure, whereas addition of LS significantly reduced systolic blood pressure, with no further effect of combined ARB and LS. Likewise, ARB had no effect on creatinine clearance, whereas creatinine clearance was decreased by LS, and was further reduced by combined ARB and LS.

Table 2 Clinical parameters during the study periods

	Regular sodium diet		Low sodium diet	
	ACEi	**ACEi+ARB**	**ACEi**	**ACEi+ARB**
General parameters				
- Systolic blood pressure (mmHg)	134 (3)	131 (3)	123 (3) * †	121 (3) * †
- Diastolic blood pressure (mmHg)	80 (2)	77 (2)	73 (2) *	71 (2) * †
- proteinuria (g/day)	1.9 (0.9-3.4)	1.6 (0.6-3.4) *	0.9 (0.5-1.7) * †	0.7 (0.4-1.4) * † ‡
- Albuminuria (mg/day)	1635 (759-3159)	1188 (500-2718) *	717 (325-1204) * †	522 (215-1259)*† ‡
- Creatinine clearance (mL/min)	69 (50-110)	72 (54-105)	67 (43-93) * †	59 (42-81) * † ‡
- Urinary creatinine excretion (mmol/day)	13.8 (0.6)	14.0 (0.5)	13.5 (0.6)	13.4 (0.6)
Sodium status				
Urinary sodium excretion (mmol/day)	189 (8)	180 (9)	106 (7) * †	105 (8) * †
Body weight (kg)	89 (3)	89 (2)	87 (2) * †	87 (2) * †
Edema n (%)	35 (8)	38 (8)	15 (6) †	8 (4) * †
Plasma sodium (mmol/L)	140.7 (0.4)	140.8 (0.4)	139.5 (0.4) * †	139.1 (0.4) * †
Plasma albumin (g/L)	38 (1)	39 (1)	40 (1) * †	40 (1) * †
Plasma total protein (g/L)	68 (1)	69 (1)	71 (1) *	72 (1) * †

Abbreviations: ACEi, ACEi inhibition; ARB, angiotensin receptor blockade; *p<0.05 vs. ACEi on regular sodium diet, †p<0.05 vs. ACEi-ARB on regular sodium diet, ‡p<0.05 vs. ACEi on low sodium diet.

Tubular injury markers during the four treatment regimens

During ACEi+RS urinary levels of NAG, KIM-1, β2MG, H-FABP, NGAL and MCP-1 were all elevated in CKD patients compared to healthy controls (Table 3). The levels of NAG (rho=0.66, p<0.001), KIM-1 (rho= 0.46, p=0.001), β2MG (rho=0.42, p=0.003) and H-FABP (rho=0.58, p<0.001) positively correlated with proteinuria during ACEi+RS. In contrast, the levels of NGAL (rho=-0.12, p=0.40) and MCP-1 (rho=0.18, p=0.22) did not correlate significantly with proteinuria during ACEi+RS.

NAG was not significantly altered by the addition of ARB, but was lowered by the addition of LS or ARB+LS to ACEi (Table 3). Likewise, KIM-1 was reduced by addition of LS or ARB+LS, but not significantly altered by the

addition of ARB. β2MG was reduced by addition of ARB, LS, or ARB+LS, to ACEi. H-FABP was reduced by the addition of ARB and further reduced by the addition of LS, with the lowest levels of H-FABP during the addition of ARB+LS to ACEi. NGAL was reduced by the addition of LS, but was not significantly altered by the addition of ARB or ARB+LS to ACEi. MCP-1 was not altered by any of the regimens.

Table 3 Urinary injury markers during 4 different treatment regimens

Healthy subjects		***CKD patients***			
		Regular sodium diet		*Low sodium diet*	
		ACEi		*ACEi*	*ACEi+ARB*
NAG (U/24h)	2.9 (2.0-4.7)	6.3 (3.0-10.9) $^{\#}$	5.0 (3.1-8.2) $^{\#}$	5.0 (3.1-8.0) $^{\#*}$	4.9 (2.5-7.1) $^{\#*}$
KIM-1 (µg/24h)	0.55 (0.35-0.97)	1.59 (1.10-2.70) $^{\#}$	1.51 (0.88-2.92) $^{\#}$	1.32 (0.71-2.31) $^{\#*\dagger}$	1.21 (0.77-2.45) $^{\#\dagger*}$
β-2-MG (µg/24h)	108 (65-166)	148 (78-2444) $^{\#}$	140 (62-712) *	136 (51-362) *	106 (57-760) *
H-FABP (µg/24h)	4 (1-6)	29 (17-94) $^{\#}$	31 (12-65) $^{\#*}$	18 (9-41) $^{\#*\dagger}$	13 (8-39) $^{\#*\dagger\ddagger}$
NGAL (µg/24h)	3 (2-3)	42 (3-65) $^{\#}$	35 (3-67) $^{\#}$	33 (4-63) $^{\#*}$	36 (4-60) $^{\#}$
MCP-1 (ng/24h)	334 (221-479)	804 (470-1276) $^{\#}$	717 (468-1069) $^{\#}$	810 (407-1200) $^{\#}$	763 (421-1448) $^{\#}$

Abbreviations: ACEi, ACEi inhibition; ARB, angiotensin receptor blockade; RS, regular sodium diet, LS, low sodium diet. $^{\#}$p<0.05 vs. healthy subjects, *p<0.05 vs. CKD on ACEi+RS, †p<0.05 vs. CKD on ACEi+ARB+RS, ‡p<0.05 vs. CKD on ACEi+LS.

Tubular injury markers according to achieved proteinuria

The number of patients that reached the proteinuria target of <0.3 g/day was largest (n=12) during combined treatment with ACEi+ARB+LS (Table 2). Individual data for this treatment period, with respect to the levels of the different tubular markers are given in figure 2 by a break-up by achieved proteinuria. For the proximal tubular injury markers NAG and β2MG and the distal tubular injury marker H-FABP, the levels were progressively lower in the patients that achieved proteinuria < 1 and < 0.3 g/day respectively. In contrast, the proximal tubular marker KIM-1 showed no differences for the different proteinuria categories, and the tubular inflammation markers NGAL

and MCP-1 were not lower in patients with a lower achieved proteinuria. Similar trends as during e.g. ACEi+ARB+LS were found during the other treatment regimens (data not shown). Underlying renal diagnoses were not essentially different between the patients groups that achieved proteinuria <0.3 (g/d) and >0.3(g/d). We further studied the subgroup of patients that reached proteinuria < 0.3 g/day during ACEi+ARB+LS, and found no differences in patient characteristics between patients in whom tubular inflammatory markers rose versus patients in whom tubular inflammatory markers decreased during ACEi+ARB+LS.

Predictors of change in tubular markers

In a multivariate analysis we investigated whether the change in urinary tubular marker excretion is associated with the change in proteinuria, blood pressure, salt intake or addition of ARB. We found that for all markers the change in tubular marker was positively correlated with the change in proteinuria, whereas for change in blood pressure the same was only true for NAG and MCP-1 excretion. In this model the treatment regimen per se (ARB or salt restricted diet) was no significant predictor of urinary marker excretion.

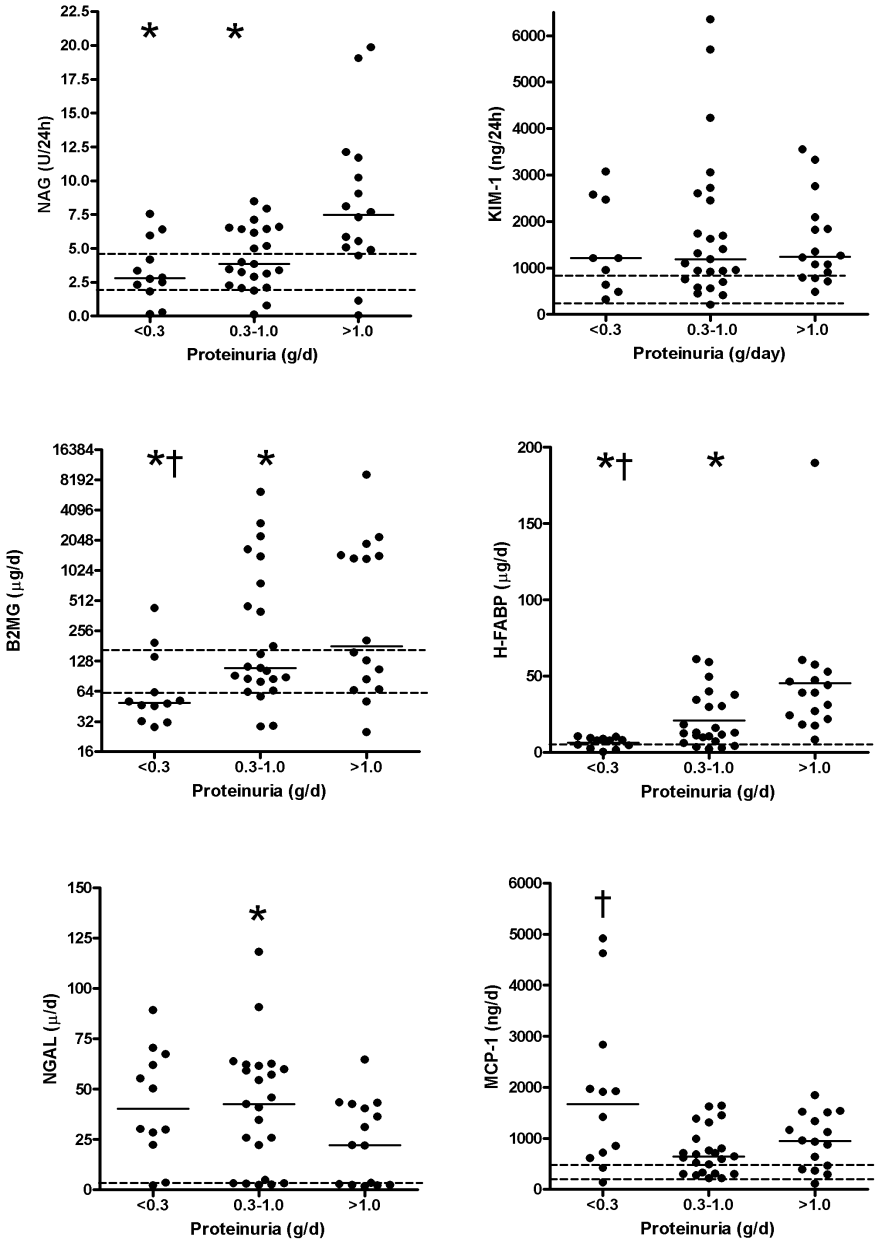


Figure 2 Individual values for urinary markers subdivided according to achieved proteinuria during combined treatment with ACE inhibition plus angiotensin receptor blockade plus low sodium diet. *p<0.05 vs. proteinuria >1.0 g/day, †p<0.05 vs. proteinuria between 0.3-1.0 g/day. The areas between dotted lines represent the interquartile range in healthy subjects.

Discussion

We found that urinary markers of proximal tubular injury (NAG, KIM-1, β2MG), tubular inflammation (MCP-1, NGAL), and remarkably also distal tubular injury (H-FABP), are elevated in non-diabetic proteinuric CKD patients despite treatment with ACEi on maximally recommended dose. At baseline, the proximal and distal tubular injury markers (NAG, KIM-1, β2MG, H-FABP) correlated with residual proteinuria, and were reduced along with reduction of proteinuria, irrespective the mode of treatment. The lowest levels of proximal and distal tubular injury markers were achieved when proteinuria fell below 0.3 g/day. In contrast, the tubular inflammation markers (MCP-1, NGAL) did not correlate with proteinuria, and remained roughly unaltered despite reduction of proteinuria.

Urinary markers of tubular injury provide a potential non-invasive tool to monitor renal damage[10;11]. We studied a broad panel of tubular markers, that reflect different aspects of renal injury in different renal compartments, and that are mediated by different processes. NAG, KIM-1 and ß2MG were measured as markers for proximal tubular damage, H-FABP was measured as a marker for distal tubular damage, and NGAL and MCP-1 were measured as inflammation markers.

All measured tubular markers were elevated in proteinuric CKD patients during monotherapy ACEi compared to healthy controls, suggesting ongoing proximal and distal tubular injury and tubular inflammation. Indeed, despite the proven benefits of monotherapy RAAS blockade, the residual renal risk remains high in CKD patients[4;39]. The elevated tubular markers in our CKD patients probably reflect this ongoing renal injury. At baseline, the proximal and distal tubular injury markers correlated with proteinuria, and were moreover reduced in proportion to the reduction of proteinuria irrespective the mode of intervention, suggesting a beneficial effect of intervention on tubular damage. Previous studies also found a tight relationship between proteinuria and proximal tubular injury markers[12;17;30;37;40;41], and a reduction of these markers by antiproteinuric therapy[12;17;42]. Our data are the first to demonstrate a similar association for H-FABP and proteinuria, including an

effect of antiproteinuric therapy. This is remarkable, as the distal tubule is classically assumed to be less sensitive to the toxic effects of urinary proteins. In line with our current findings, recent other data challenge this latter assumption[30;41].

In contrast, the tubular inflammation markers (MCP-1, NGAL) remained roughly unaltered despite reduction of proteinuria irrespective of the mode of intervention. This was unexpected, as reduction of proteinuria is assumed to protect the tubulointerstitium by amelioration of the proinflammatory effects of leaked proteins[43-45]. Whereas the latter assumption is supported by our findings on NAG, KIM-1, ß2MG and H-FABP, their reductions dissociate from the lack of effect on tubular inflammation makers. It cannot be excluded that in our study the interventions were not rigorous enough, or that six weeks of treatment was too short for an anti-inflammatory effect to become apparent. Alternatively, tubular inflammation as reflected by MCP-1 and NGAL may not have been exclusively proteinuria-driven, which would be in line with the absence of a correlation between MCP-1, NGAL and proteinuria in these patients. Possibly, anti-inflammatory effects of a reduction in proteinuria per se may have been offset by reactive increases in renin or aldosterone, which can exert proinflammatory effects.[45;51;52] Others also reported absence of a correlation between MCP-1 and proteinuria in renal patients without (high-grade) inflammatory nephropathy treated with RAAS blockade.[46;47] However, a correlation between (a change in) MCP-1 and proteinuria was present in patients with inflammatory nephropathy and in renal patients that were treated with immunosuppressive therapy, antibiotics, or oral antidiabetics.[48-50] Hence, the presence of a relationship between (change in) MCP-1 and proteinuria may be dependent on renal diagnosis and the mode of treatment. Of note, we found no clear association between the various histological diagnoses and the level of tubular or inflammatory markers in our study.

The levels of proximal and distal tubular injury markers were lowest in patients in whom proteinuria levels fell below 0.3 g/day. This is in line with previous, principally observational, data suggesting that proteinuria below 0.3 g/day is associated with a better renal outcome.[53;6] These data can be

considered to support the notion that the current treatment target for proteinuria to below 1.0 g/day is too liberal and that titration of treatment to obtain proteinuria to levels below 0.3 g/day may be needed for optimal renoprotection[53;6]. However, such a conclusion should be taken with caution. First, the discrepancies between the level of inflammatory markers and residual proteinuria indicate that ongoing tubular damage is a complex process. Second, it is also not known whether specific titration of residual proteinuria to below 0.3 g/day will improve urinary tubular marker profile and outcome, or whether the better reduction of tubular injury markers in subjects in whom proteinuria fell below 0.3 g/day simply reflects a more benign phenotype. It remains to be proven by prospective intervention studies whether our short-term findings translate into long-term renal outcome, in other words, whether patients with treatment titration to proteinuria below 0.3 g/day and low levels of tubular injury markers have slower -or even absent- progression to end stage renal disease.

The strengths of this study are that we measured not a single tubular injury marker, but a broad panel that reflect diverse aspects of tubular injury and are mediated by different processes. We studied not only the cross-sectional association between tubular injury markers and proteinuria, but also the effect of interventions. Furthermore all samples were measured in one run, thus avoiding interassay variation. The major limitations of this study are, first, that it is a post-hoc analysis, and second, that it provides short term data only. The impact of our data for long-term outcome will require separate study.

In conclusion, urinary markers of proximal and distal tubular injury, and tubular inflammation are elevated in non-diabetic proteinuric CKD patients with persistent proteinuria despite maximal ACEi, probably reflecting ongoing renal injury. Proximal and distal tubular injury markers are reduced along with intensified reduction of proteinuria by combinations of ACEi, ARB and low sodium diet. Lowest values were obtained during the combination of ACEi, ARB and sodium restriction, pleading for prescription of such a regimen to achieve optimal long-term renoprotection. However, markers of

tubular inflammation remained largely unaffected and, if anything, increased in patients with the lowest proteinuria values. Long-term prospective intervention studies should investigate therefore whether titration of treatment to achieve proteinuria levels below 0.3 g/day will further improve long-term renal outcome.

Acknowledgements
None.

Reference List

1. Jafar TH, Stark PC, Schmid CH *et al*. Progression of chronic kidney disease: the role of blood pressure control, proteinuria, and angiotensin-converting enzyme inhibition: a patient-level meta-analysis. *Ann Intern Med* 2003; 139: 244-252

2. Ruggenenti P, Schieppati A, Remuzzi G. Progression, remission, regression of chronic renal diseases. *Lancet* 2001; 357: 1601-1608

3. Lewis EJ, Hunsicker LG, Bain RP, Rohde RD. The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. The Collaborative Study Group. *N Engl J Med* 1993; 329: 1456-1462

4. Brenner BM, Cooper ME, de Zeeuw D *et al*. Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 2001; 345: 861-869

5. Cianciaruso B, Bellizzi V, Minutolo R *et al*. Salt intake and renal outcome in patients with progressive renal disease. *Miner Electrolyte Metab* 1998; 24: 296-301

6. Ruggenenti P, Perticucci E, Cravedi P *et al*. Role of remission clinics in the longitudinal treatment of CKD. *J Am Soc Nephrol* 2008; 19: 1213-1224

7. Ruggenenti P, Perna A, Remuzzi G. Retarding progression of chronic renal disease: the neglected issue of residual proteinuria. *Kidney Int* 2003; 63: 2254-2261

8. Nath KA. Tubulointerstitial changes as a major determinant in the progression of renal damage. *Am J Kidney Dis* 1992; 20: 1-17

9. Deelman L, Sharma K. Mechanisms of kidney fibrosis and the role of antifibrotic therapies. *Curr Opin Nephrol Hypertens* 2009; 18: 85-90

10. Waanders F, Navis G, van Goor H. Urinary tubular biomarkers of kidney damage: potential value in clinical practice. *Am J Kidney Dis* 2010; 55: 813-816

111. D'Amico G, Bazzi C. Urinary protein and enzyme excretion as markers of tubular damage. *Curr Opin Nephrol Hypertens* 2003; 12: 639-643

12. Waanders F, Vaidya VS, van Goor H *et al*. Effect of renin-angiotensin-aldosterone system inhibition, dietary sodium restriction, and/or diuretics on urinary kidney injury molecule 1 excretion in nondiabetic proteinuric kidney disease: a post hoc analysis of a randomized controlled trial. *Am J Kidney Dis* 2009; 53: 16-25

13. K/DOQI clinical practice guidelines on hypertension and antihypertensive agents in chronic kidney disease. *Am J Kidney Dis* 2004; 43: S1-290

14. Moderate dietary sodium restriction added to ACE inhibition is effective than dual blockade in lowering blood pressure and proteinuria: results from a randomised controlled trial; Slagman MC, Waanders F, Hemmelder MH, Woittiez AJ, Janssen WMT, Lambers Heerspink H, Navis GJ, Laverman GD; BMJ; 2011 in press

15. Wolkow PP, Niewczas MA, Perkins B *et al*. Association of urinary inflammatory markers and renal decline in microalbuminuric type 1 diabetics. *J Am Soc Nephrol* 2008; 19: 789-797

16. Kern EF, Erhard P, Sun W, Genuth S, Weiss MF. Early urinary markers of diabetic kidney disease: a nested case-control study from the Diabetes Control and Complications Trial (DCCT). *Am J Kidney Dis* 2010; 55: 824-834

17. Bazzi C, Petrini C, Rizza V *et al*. Urinary N-acetyl-beta-glucosaminidase excretion is a marker of tubular cell dysfunction and a predictor of outcome in primary glomerulonephritis. *Nephrol Dial Transplant* 2002; 17: 1890-1896

18. Ichimura T, Bonventre JV, Bailly V *et al*. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. *J Biol Chem* 1998; 273: 4135-4142

19. van Timmeren MM, van den Heuvel MC, Bailly V, Bakker SJ, van GH, Stegeman CA. Tubular kidney injury molecule-1 (KIM-1) in human renal disease. *J Pathol* 2007; 212: 209-217

20. Waanders F, van Timmeren MM, Stegeman CA, Bakker SJ, van GH. Kidney injury molecule-1 in renal disease. *J Pathol* 2010; 220: 7-16

21. Bonventre JV. Kidney injury molecule-1 (KIM-1): a urinary biomarker and much more. *Nephrol Dial Transplant* 2009; 24: 3265-3268

22. Liangos O, Perianayagam MC, Vaidya VS *et al*. Urinary N-acetyl-beta-(D)-glucosaminidase activity and kidney injury molecule-1 level are associated with adverse outcomes in acute renal failure. *J Am Soc Nephrol* 2007; 18: 904-912

23. van Timmeren MM, Vaidya VS, van Ree RM *et al*. High urinary excretion of kidney injury molecule-1 is an independent predictor of graft loss in renal transplant recipients. *Transplantation* 2007; 84: 1625-1630

24. Vaidya VS, Waikar SS, Ferguson MA *et al*. Urinary biomarkers for sensitive and specific detection of acute kidney injury in humans. *Clin Transl Sci* 2008; 1: 200-208

25. Drueke TB, Massy ZA. Beta2-microglobulin. *Semin Dial* 2009; 22: 378-380

26. Gerritsen KG, Peters HP, Nguyen TQ *et al*. Renal proximal tubular dysfunction is a major determinant of urinary connective tissue growth factor excretion. *Am J Physiol Renal Physiol* 2010; 298: F1457-F1464

27. Branten AJ, du Buf-Vereijken PW, Klasen IS *et al*. Urinary excretion of beta2-microglobulin and IgG predict prognosis in idiopathic membranous nephropathy: a validation study. *J Am Soc Nephrol* 2005; 16: 169-174

28. Maatman RG, Van Kuppevelt TH, Veerkamp JH. Two types of fatty acid-binding protein in human kidney. Isolation, characterization and localization. *Biochem J* 1991; 273 (Pt 3): 759-766

29. Pelsers MM. Fatty acid-binding protein as marker for renal injury. *Scand J Clin Lab Invest Suppl* 2008; 241: 73-77

30. Hofstra JM, Deegens JK, Steenbergen EJ, Wetzels JF. Urinary excretion of fatty acid-binding proteins in idiopathic membranous nephropathy. *Nephrol Dial Transplant* 2008;

31. Bonventre JV, Vaidya VS, Schmouder R, Feig P, Dieterle F. Next-generation biomarkers for detecting kidney toxicity. *Nat Biotechnol* 2010; 28: 436-440

32. Kuwabara T, Mori K, Mukoyama M *et al*. Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons. *Kidney Int* 2009; 75: 285-294

33. Mishra J, Ma Q, Prada A *et al*. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *J Am Soc Nephrol* 2003; 14: 2534-2543

34. Bolignano D, Lacquaniti A, Coppolino G *et al*. Neutrophil gelatinase-associated lipocalin (NGAL) and progression of chronic kidney disease. *Clin J Am Soc Nephrol* 2009; 4: 337-344

35. Stangou M, Alexopoulos E, Papagianni A *et al*. Urinary levels of epidermal growth factor, interleukin-6 and monocyte chemoattractant protein-1 may act as predictor markers of renal function outcome in immunoglobulin A nephropathy. *Nephrology (Carlton)* 2009; 14: 613-620

36. Fornoni A, Ijaz A, Tejada T, Lenz O. Role of inflammation in diabetic nephropathy. *Curr Diabetes Rev* 2008; 4: 10-17

37. Tam FW, Riser BL, Meeran K, Rambow J, Pusey CD, Frankel AH. Urinary monocyte chemoattractant protein-1 (MCP-1) and connective tissue growth factor (CCN2) as prognostic markers for progression of diabetic nephropathy. *Cytokine* 2009; 47: 37-42

38. Camilla R, Brachemi S, Pichette V *et al*. Urinary monocyte chemotactic protein 1: marker of renal function decline in diabetic and nondiabetic proteinuric renal disease. *J Nephrol* 2011; 24: 60-67

39. Lewis EJ, Hunsicker LG, Bain RP, Rohde RD. The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. The Collaborative Study Group. *N Engl J Med* 1993; 329: 1456-1462

40. Eardley KS, Zehnder D, Quinkler M *et al*. The relationship between albuminuria, MCP-1/CCL2, and interstitial macrophages in chronic kidney disease. *Kidney Int* 2006; 69: 1189-1197

41. Nauta FL, Boertien WE, Bakker SJ *et al*. Glomerular and tubular damage markers are elevated in patients with diabetes. *Diabetes Care* 2011; 34: 975-981

42. Kasahara M, Mori K, Satoh N *et al*. Reduction in urinary excretion of neutrophil gelatinase-associated lipocalin by angiotensin receptor blockers in hypertensive patients. *Nephrol Dial Transplant* 2009; 24: 2608-2609

43. Abbate M, Zoja C, Remuzzi G. How does proteinuria cause progressive renal damage? *J Am Soc Nephrol* 2006; 17: 2974-2984

44. Wang SN, LaPage J, Hirschberg R. Role of glomerular ultrafiltration of growth factors in progressive interstitial fibrosis in diabetic nephropathy. *Kidney Int* 2000; 57: 1002-1014

45. Zoja C, Garcia PB, Remuzzi G. The role of chemokines in progressive renal disease. *Front Biosci* 2009; 14: 1815-1822

46. Vaidya VS, Niewczas MA, Ficociello LH *et al*. Regression of microalbuminuria in type 1 diabetes is associated with lower levels of urinary tubular injury biomarkers, kidney injury molecule-1, and N-acetyl-beta-D-glucosaminidase. *Kidney Int* 2011; 79: 464-470

47. Camilla R, Brachemi S, Pichette V *et al*. Urinary monocyte chemotactic protein 1: marker of renal function decline in diabetic and nondiabetic proteinuric renal disease. *J Nephrol* 2011; 24: 60-67

48. Wasilewska A, Zoch-Zwierz W, Taranta-Janusz K, Kolodziejczyk Z. Urinary monocyte chemoattractant protein-1 excretion in children with glomerular proteinuria. *Scand J Urol Nephrol* 2011; 45: 52-59

49. Tone A, Shikata K, Nakagawa K, Hashimoto M, Makino H. Renoprotective effects of clarithromycin via reduction of urinary MCP-1 levels in type 2 diabetic patients. *Clin Exp Nephrol* 2011; 15: 79-85

50. Hu YY, Ye SD, Zhao LL, Zheng M, Wu FZ, Chen Y. Hydrochloride pioglitazone decreases urinary cytokines excretion in type 2 diabetes. *Clin Endocrinol (Oxf)* 2010; 73: 739-743

51. Hollenberg NK. Direct renin inhibition and the kidney. *Nat Rev Nephrol* 2010; 6: 49-55

52. Slagman MC, Navis G, Laverman GD. Dual blockade of the renin-angiotensin-aldosterone system in cardiac and renal disease. *Curr Opin Nephrol Hypertens* 2010; 19: 140-152

53. Peterson JC, Adler S, Burkart JM *et al*. Blood pressure control, proteinuria, and the progression of renal disease. The Modification of Diet in Renal Disease Study. *Ann Intern Med* 1995; 123: 754-762

Chapter 8

Thesis summary

Summary

Chronic kidney disease is recognized as a major global public health problem.[1;2] The disease affects 10–16% of the adult population in Asia, Australia, Europe, and the USA.[3-6] and increases the risk of all-cause mortality, cardiovascular disease, and progression to kidney failure, even after accounting for traditional risk factors such as hypertension and diabetes mellitus.[1;7] Chronic kidney disease is defined as persistent kidney damage, usually marked by albuminuria and reduced glomerular filtration rate (GFR). Recently, a meta-analysis of 21 studies has been published investigating the combined predictive value of both albuminuria and eGFR on cardiovascular and all-cause mortality in the general population.[8] It was shown that albuminuria and eGFR are independently of each other associated with cardiovascular and all-cause mortality. On a smaller scale it has also been shown that low eGFR and higher albuminuria predict renal function decline in chronic kidney disease[9-11], in high risk population (defined by presence of diabetes, hypertension or a cardiovascular disease history), as well as in the general population.[12-14] This can be important since albuminuria is also a predictor of outcome when eGFR is still normal. Detection of CKD in an early stage offers possibilities to start early treatment and thereby to delay or prevent complications of CKD.[15]

The question arises why albuminuria adds to GFR to predict renal prognosis? Albuminuria is the end result of glomerular filtration and tubular reabsorption of albumin.[16;17] Increased urinary albumin excretion can therefore be the result of damaged glomeruli or tubulointerstitial damage. GFR and albuminuria independently of each other predict outcomes. Since GFR represents glomerular function, this suggests that albuminuria is the result of tubular reuptake dysfunction. Therefore it might be that specific markers of tubular damage outperform albuminuria or add to the predictive capacity of albuminuria to predict renal prognosis. In acute kidney disease it has been demonstrated that specific urinary markers of tubular damage or inflammation can predict the onset of kidney failure even before an increase

in serum creatinine is observed.[18;19] Until recently, little research has been done regarding the value of these markers in chronic kidney disease and in the general population. How can these markers be of aid? Several purposes for use can be thought of. First, these markers could be of help in identifying patients with early tubulo-interstitial damage. Second, these markers could be useful in predicting renal prognosis. Third, they could potentially be used as markers to monitor the effect of medication.

As also stated in the introduction of this thesis, we have measured markers of the glomerulus, proximal tubule, distal tubule and general inflammation, both in urine as in plasma. Urinary markers of renal injury are elevated during glomerular and tubulointerstitial injury and provide a non-invasive tool to monitor renal damage. In the studies described in this thesis we measured IgG and IgG-4 as glomerular markers. Urinary loss of the positively charged IgG (150 kDa) is associated with loss of size selectivity of the glomerular basement membrane, whereas urinary loss of the negatively IgG-4 is considered to reflect loss of charge selectivity.[20]

The tubular injury markers investigated here reflect injury in different parts of the renal tubule and are mediated by different processes. KIM-1, NAG, Cystatine C and ß-2-microglobuline were measured as markers for proximal tubular damage and H-FABP as marker for distal tubular damage. KIM-1 is selectively expressed by injured proximal tubular cells.[21] KIM-1 expression is related to tubulointerstitial damage and correlates with the severity of kidney function impairment.[22] NAG (135 kDa) is a lysosymal enzyme that is predominantly produced in the proximal tubule, and released into urine upon cellular damage. Cystatin C is an endogenous cysteine proteinase inhibitor, produced by most nucleated cells at a relatively constant rate and released into the plasma. In normal renal function, tubular reabsorption and catabolism of cystatin C is almost complete and cystatin C is thus only detectable in very small quantities in the urine.[23] β-2-microglobulin (12 kDa) is a component of MHC class 1 molecules, which are present on all

nucleated cells. B-2-microglobulin is freely filtered through the glomerulus and subsequently reabsorbed by proximal tubular cells. Urinary β-2-microglobulin is a marker of proximal tubular reabsorption incapacity. H-FABP was measured as marker of distal tubular damage. H-FABP (15kDa) is an intracellular carrier protein that is present in cytoplasm of distal tubular cells. Urinary H-FABP results from release by structurally damaged tubular cells.[24;25]

NGAL and MCP-1 were measured as markers of general inflammation. NGAL (25 kDa) is expressed by neutrophils and other epithelial cells. It was found to reflect damage to glomeruli, and proximal and distal tubules.[26-28] MCP-1 (13-30 kDa) is expressed by inflammatory cells such as monocytes, and also by resident renal cells, i.e. mesangial, endothelial, and tubular epithelial cells.[29] Renal cells produce MCP-1 in response to a variety of pro-inflammatory stimuli.[30]

In **chapter 2** these glomerular and tubular damage markers were measured in a cohort of 94 patients with diabetes mellitus with normo-, micro- and macro-albuminuria. As mentioned above, albuminuria is the end result of glomerular filtration and tubular reabsorption. It is increasingly appreciated that the renal interstitium plays a role in the pathogenesis of diabetic nephropathy, as the consequence of a prolonged exposure to hemodynamic and metabolic injuring factors associated with diabetes mellitus.[17;31] Furthermore, persistent albuminuria secondary to glomerular lesions may be directly harmful to renal tubular cells, leading to tubular inflammation and fibrosis.[32;33] In these patients we found that urinary excretion of all markers was higher when compared to non-diabetic healthy controls. Interestingly, this was already found in the normo-albuminuric diabetic, who had an eGFR and albuminuria comparable to the healthy controls. This suggests that even in diabetic patients without any sign of renal damage according to prevailing guidelines, already some kind of ‘tubular stress’ is evident. This indicates that the investigated markers may be very sensitive damage markers, being

already elevated in a very early stage of the disease. Furthermore, almost all tubular damage markers were found to be significantly associated with the degree of renal damage, assessed as eGFR and albuminuria. This suggests that they might be fit to predict renal function in a longitudinal study. Of all 5 investigated urinary tubular markers, the distal tubular marker H-FABP was the only one that was significantly associated with eGFR, independent of albuminuria. This renders especially H-FABP a promising sensitive marker of early diabetic kidney disease.

In this study, we measured all damage markers in fresh urine samples. Previously it has been shown that frozen storage of urine samples during 1 year is associated with a decrease in urinary albumin concentration, with a wide variation between urine samples in the amount to which albumin decreases.[34;35] These storage effects are of importance. In epidemiological research albuminuria is often measured in urine samples that have been stored for prolonged periods of time. It has been shown that the value of urinary albumin excretion to predict mortality is considerably better when assessed in fresh urine samples in comparison to when assessed in the same samples after prolonged frozen storage.[36] In a previous study it was demonstrated that urinary pH is a determinant of the decline in urinary albumin concentration during frozen storage at -20 ºC, with the largest decline at pH 5.0, whereas albumin in urine with pH 8.0 showed on average no decline.[37]

Therefore we investigated in **chapter 3** whether alkalinisation of urine samples before freezing preserves albumin concentration. 90 urine samples of patients with diabetes mellitus visiting a diabetes outpatient clinic were collected and divided into 2 portions. The first portion was adjusted to pH > 8 and the second was left unprocessed. Albumin concentration was assessed in these fresh samples and after 7 days, 1, 6 and 12 months of frozen storage at -20 ºC and -80 ºC. We found that pH adjustment to > 8 prevents decline and reduces increase in variability of albumin concentration in

samples that were stored frozen for 12 months at -20 ºC compared to pH unadjusted urine samples also stored at -20 ºC. However, no difference in average decline in urinary albumin concentration between unadjusted samples stored at -80 ºC and pH adjusted samples stored at -20 ºC was found. This renders pH adjustment to values >8 of urine samples and consequent storage at -20 ºC as a good alternative for frozen storage at -80 ºC.

Since we planned to measure in our studies not only urinary albumin concentration, but also other urinary renal damage markers, and as yet little is known about the effect of frozen storage average decline and variability of urine concentration of these markers, we investigated in **chapter 4** the effect of frozen storage on a panel of specific damage markers of the glomerulus, proximal tubule and distal tubule, and general inflammation markers. First, we found that besides cystatin C all urinary markers decrease in concentration and show an increase in variability after one year of frozen storage. Second, we stored urine samples under several different storage conditions to see whether this could prevent or reduce a decline in concentration. We alkalized urine samples before or after frozen storage or added protease inhibitors and stored samples for 1 year at -20 and -80 ºC. Unfortunately, none of the investigated protocols could prevent or reduce the decline or increase in variability that is observed with the various damage markers. Third, we investigated the correlation between fresh measured concentrations and concentrations measured after frozen storage. All marker concentrations assessed from frozen samples showed significant correlations to the freshly measured concentrations. Given these observations we conclude that, if possible, these markers are preferably measured in fresh urine samples. However, since these marker concentrations from frozen samples are still significantly correlated to concentrations from fresh samples, we feel that results can be used to predict end points. However, results obtained from frozen samples should be interpreted with caution, since absence of a significant relation with the

endpoint under investigation can be due to absence of this relation, or due to storage effects.

As discussed above it may be that urinary markers of tubular damage predict renal outcome in addition to albuminuria. Therefore it is investigated in **chapter 5** in frozen urine samples obtained from patients more than 1 year after renal transplantation whether specific markers of tubular damage predict graft failure or decline in renal function. The most common used prognostic urinary marker in chronic transplant dysfunction is total proteinuria. Proteinuria is not only the loss of albumin, but consists of a wide variety of proteins. Of note, chronic transplant dysfunction is characterised by tubular histologic changes, such as interstitial fibrosis and tubular atrophy.[38;39] It has even been demonstrated that tubular histologic damage is better associated with renal prognosis than glomerular damage.[40] Therefore we measured specific markers of tubular damage. Since it can take considerable time for a person with chronic transplant dysfunction to progress to graft failure, we did not only look at graft failure (only n=42 of 606 patients), but also at loss of renal function as an endpoint.[41;42] We showed after a median follow-up of 4.7 years that all tubular damage markers predicted graft failure, but not independent of proteinuria or albuminuria. Of the 4 investigated tubular markers KIM-1 and H-FABP also predicted change in eGFR. However, we found that albuminuria predicts both graft failure and change in eGFR better than all investigated tubular damage marker and better than proteinuria. As Kasiske mentioned in an editorial about this study, besides numerous strengths, also some limitations should be considered.[43] For example, measurements were made only once and at variable times after kidney transplant. Results could have been different if measurements were made at the same (and preferably multiple) times post-transplant in all patients. A limited number of studies recently also investigated the value of urinary tubular damage markers in patients after transplantation to predict renal prognosis. In a cohort of 145 kidney transplant recipients it was shown that urinary KIM-1 is a powerful predictor

of graft failure.[44] In this larger cohort we corroborated that KIM-1 is a predictor of graft failure, and also of renal function loss, adding information that the predictive value is not independent of albuminuria. One other study in patients with a stable graft showed that subclinical tubulitis is associated with higher levels of urinary NGAL.[45] The finding that albuminuria outperforms proteinuria is in line with the study of Halimi[46], which also showed that albuminuria is a good predictor of graft loss, even in nonproteinuric patients. This study measured in 616 patients albuminuria and proteinuria. They looked at graft failure and all cause mortality with a median follow-up of 3.3 years. In contrast, in our study we compared in a relatively large cohort with a median follow-up of 4.7 years the predictive value of 4 tubular markers in addition to proteinuria and albuminuria. Furthermore, since graft failure and death occur only in a limited amount of patients, we also investigated loss of renal function.

We have investigated in patients with chronic kidney disease urinary levels of tubular damage markers in chapters 2 and 5. However, very little is known about the potential value of these markers to predict renal outcome in the general population. This is an important issue since early identification of subjects at high risk for CKD progression offers possibilities to start early treatment and thereby to delay or prevent complications of CKD.[15] Therefore we investigated in **chapter 6** in subjects participating in the PREVEND study how urinary glomerular and tubular damage markers at baseline are related to progression of renal damage, measured as increase in albuminuria during follow-up. Progression of albuminuria during follow-up is particularly important, since a rise in albuminuria has been shown to be associated with a more rapid decline in renal function and increased mortality in patients with diabetes[47] and in subjects from the general population with a higher risk for cardiovascular events.[48] As in the introduction of this thesis already has been stated, albuminuria is the result of both glomerular leakage and tubular reabsorption. We hypothesized that tubular damage may be of help to predict an increase in albuminuria during follow-up. To investigate this

hypothesis we selected from the PREVEND cohort of 8592 participants those subjects without renal disease at baseline, and with the 20% most progression in albuminuria per year and albuminuria at the last follow-up visit >150 mg/24h. This yielded 183 subjects. These subjects were matched on sex, age and baseline albuminuria to controls (in a 1:2 ratio) that did not show such an increase in albuminuria. We showed that progressors in albuminuria in comparison to controls had higher urinary excretion of the glomerular marker IgG, but lower excretion of tubular damage markers KIM-1, NAG, cystatine C, beta-2-microglobulin and the general inflammation markers NGAL and MCP-1. Possibly because our matching criteria –we did not only match on age and sex, but also on baseline albuminuria- we did not find that higher proximal tubular damage markers were associated with progression in albuminuria during follow-up.

These data suggest that albuminuria increases in subjects with albuminuria associated with glomerular damage, whereas subjects with albuminuria associated with a tubular reabsorption defect do not show an increase in albuminuria. Second, we measured all plasma concentrations of the investigated markers, to investigate whether plasma concentrations influence the urinary excretion of these markers. Therefore fractional excretions of all investigated damage markers were calculated. We found that the fractional excretion of IgG was higher in subjects with progressive albuminuria, whereas the fractional excretion of the tubular damage markers was lower in these subjects. This analysis thus yielded similar results as studying only the urinary excretion of these markers, corroborating our conclusion that tubular albuminuria in these subjects is les harmfull compared to glomerular albuminuria. How do these data relate to current literature? It is known for a number of tubular damage markers that they predict progression of chronic kidney disease assessed as decline in renal function[49-52], but very little studies have looked into albuminuria, the early marker of kidney damage. Vaidya et al have reported that in patients with diabetes mellitus a spontaneous regression of albuminuria is associated with

lower urinary excretion of NAG and KIM-1.[53] Urinary NAG predicts the subsequent occurrence of albuminuria in diabetic patients.[54] We are to our knowledge the first to investigate these markers in a cohort of subjects obtained from the general population.

In both chapter 6 and chapter 7 we investigated the value of a panel of biomarkers to predict renal prognosis. These markers however can not only be used to predict prognosis, but also to asses on a short-term basis the potential renoprotective effect of medication, and in an individual to titrate alleged renoprotective medication. Therefore we investigated in **chapter 7** the effect of dietary sodium restriction and addition of an angiotensin II receptor blocker (ARB) on urinary renal damage markers in 52 non-diabetic patients with chronic kidney disease with significant residual proteinuria during ACE inhibition. Patients were enrolled in 4 treatment periods of 6 weeks in random order. Treatment periods consisted of ACE inhibition on maximal dose plus placebo and ACE inhibition plus angiotensin receptor blockers (ARB) on supramaximal dose (Valsartan 320 mg/d). Both treatments were consecutively combined with a low sodium diet and a regular sodium diet. 24 hour urine was collected at the end of each 6 week treatment period and stored frozen at -80 ºC. Damage markers were measured in these samples. We found that the effect of sodium restriction on lowering tubular damage markers is more outspoken than addition of an ARB. Furthermore, we investigated whether more intensive treatment targets for proteinuria are followed by equal decline in tubular damage markers. We observed that the investigated markers do not decline proportionally to proteinuria, which would be expected, since proteinuria itself is supposed to be toxic to epithelial tubular cells. This suggests ongoing tubular damage, even when current treatment targets for proteinuria are met.

Future perspectives

In this thesis we show that determination of urinary excretion of renal damage markers holds great potential. Several issues still have to be clarified in future research. First, until now, most studies have investigated whether these markers are associated with kidney damage (assessed as eGFR or albuminuria) only in cross-sectional studies. Only a few prospective studies are now being published showing that some markers also predict renal prognosis in diabetic kidney disease.[54-56] At this moment, however, is unknown whether these biomarkers will outperform or add to measurement of the biomarker that is presently used to define and predict chronic kidney disease, being albuminuria. Furthermore, almost all studies investigating the predictive value of these markers are of retrospective design, rendering these studies vulnerable for several sources of bias. One of them is that markers in general are measured in urine samples that have been stored frozen for prolonged periods in time. We showed that such storage is associated with a decline in marker concentration and an increase in variability. This will influence the predictive value of these markers insofar that dilution of effect size may be anticipated. To overcome this problem, prospective longitudinal studies should be conducted, preferably using fresh urine samples.

Second, most assays used have a considerable inter- and intra-assay variability. Improvement of assay performance is therefore necessary. Several companies offer nowadays assays for the same marker probably aiming for different epitopes on the proteins to be measured. This can result in differences in damage marker concentrations in the same sample, which makes it difficult to compare the results of studies that use different assays. Standardization of assays for the various markers that are presently used still has to be done.

Another point is that, obviously, pathological processes differ between different renal diseases. It could therefore well be that in other conditions

tubular damage markers do may be of help. Depending on the disease under investigation, it is most probable that a panel of urinary damage markers will have the best predictive capacity to predict progression of kidney disease. Research at this moment is merely focussed on identifying single markers. Future research should therefore focus not only on one marker at a time, but at panels of markers, that wil probably differ between patients groups and clinical characteristics.

Clarifying these issues will necessitate large studies in well defined patient groups, using rigid methodology with respect to storage of urine samples and assay quality. Such studies will be of help to clarify whether certain novel urinary markers or combinations of markers can reliably predict renal prognosis. Until such studies are performed the urinary biomarker to be measured remains albuminuria.

Reference List

1. Levey AS, Atkins R, Coresh J *et al*. Chronic kidney disease as a global public health problem: approaches and initiatives - a position statement from Kidney Disease Improving Global Outcomes. *Kidney Int* 2007; 72: 247-259

2. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Am J Kidney Dis* 2002; 39: S1-266

3. Wen CP, Cheng TY, Tsai MK *et al*. All-cause mortality attributable to chronic kidney disease: a prospective cohort study based on 462 293 adults in Taiwan. *Lancet* 2008; 371: 2173-2182

4. Chadban SJ, Briganti EM, Kerr PG *et al*. Prevalence of kidney damage in Australian adults: The AusDiab kidney study. *J Am Soc Nephrol* 2003; 14: S131-S138

5. Hallan SI, Coresh J, Astor BC *et al*. International comparison of the relationship of chronic kidney disease prevalence and ESRD risk. *J Am Soc Nephrol* 2006; 17: 2275-2284

6. Coresh J, Selvin E, Stevens LA *et al*. Prevalence of chronic kidney disease in the United States. *JAMA* 2007; 298: 2038-2047

7. Sarnak MJ, Levey AS, Schoolwerth AC *et al*. Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. *Circulation* 2003; 108: 2154-2169

8. Matsushita K, van der Velde M, Astor BC *et al*. Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. *Lancet* 2010; 375: 2073-2081

9. Astor BC, Matsushita K, Gansevoort RT *et al*. Lower estimated glomerular filtration rate and higher albuminuria are associated with mortality and end-stage renal disease. A collaborative meta-analysis of kidney disease population cohorts. *Kidney Int* 2011;

10. Ninomiya T, Perkovic V, de Galan BE *et al*. Albuminuria and kidney function independently predict cardiovascular and renal outcomes in diabetes. *J Am Soc Nephrol* 2009; 20: 1813-1821

11. Tangri N, Stevens LA, Griffith J *et al*. A predictive model for progression of chronic kidney disease to kidney failure. *JAMA* 2011; 305: 1553-1559

12. Hemmelgarn BR, Manns BJ, Lloyd A *et al*. Relation between kidney function, proteinuria, and adverse outcomes. *JAMA* 2010; 303: 423-429

13. Verhave JC, Gansevoort RT, Hillege HL, Bakker SJ, de ZD, de Jong PE. An elevated urinary albumin excretion predicts de novo development of renal function impairment in the general population. *Kidney Int Suppl* 2004; S18-S21

14. van der Velde M, Halbesma N, de Charro FT *et al*. Screening for albuminuria identifies individuals at increased renal risk. *J Am Soc Nephrol* 2009; 20: 852-862

15. Ruggenenti P, Perna A, Remuzzi G. ACE inhibitors to prevent end-stage renal disease: when to start and why possibly never to stop: a post hoc analysis of the REIN trial results. Ramipril Efficacy in Nephropathy. *J Am Soc Nephrol* 2001; 12: 2832-2837

16. Bakker SJ, Gansevoort RT, de Zeeuw D. Albuminuria: what can we expect from the determination of nonimmunoreactive albumin? *Curr Hypertens Rep* 2009; 11: 111-117

17. Comper WD, Hilliard LM, Nikolic-Paterson DJ, Russo LM. Disease-dependent mechanisms of albuminuria. *Am J Physiol Renal Physiol* 2008; 295: F1589-F1600

18. Bagshaw SM, Langenberg C, Haase M, Wan L, May CN, Bellomo R. Urinary biomarkers in septic acute kidney injury. *Intensive Care Med* 2007; 33: 1285-1296

19. Trof RJ, Di MF, Leemreis J, Groeneveld AB. Biomarkers of acute renal injury and renal failure. *Shock* 2006; 26: 245-253

20. Hemmelder MH, de Zeeuw D, de Jong PE. Measurement of glomerular charge selectivity in non-diabetic renal disease. *Nephrol Dial Transplant* 1997; 12 Suppl 2: 57-62

21. Waanders F, Navis G, van Goor H. Urinary tubular biomarkers of kidney damage: potential value in clinical practice. *Am J Kidney Dis* 2010; 55: 813-816

22. Waanders F, van Timmeren MM, Stegeman CA, Bakker SJ, van Goor H. Kidney injury molecule-1 in renal disease. *J Pathol* 2010; 220: 7-16

23. Herget-Rosenthal S, Feldkamp T, Volbracht L, Kribben A. Measurement of urinary cystatin C by particle-enhanced nephelometric immunoassay: precision, interferences, stability and reference range. *Ann Clin Biochem* 2004; 41: 111-118

24. Maatman RG, Van Kuppevelt TH, Veerkamp JH. Two types of fatty acid-binding protein in human kidney. Isolation, characterization and localization. *Biochem J* 1991; 273 (Pt 3): 759-766

25. Pelsers MM. Fatty acid-binding protein as marker for renal injury. *Scand J Clin Lab Invest Suppl* 2008; 241: 73-77

26. Mishra J, Ma Q, Prada A *et al*. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *J Am Soc Nephrol* 2003; 14: 2534-2543

27. Kuwabara T, Mori K, Mukoyama M *et al*. Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons. *Kidney Int* 2009; 75: 285-294

28. Bonventre JV, Vaidya VS, Schmouder R, Feig P, Dieterle F. Next-generation biomarkers for detecting kidney toxicity. *Nat Biotechnol* 2010; 28: 436-440

29. Stangou M, Alexopoulos E, Papagianni A *et al*. Urinary levels of epidermal growth factor, interleukin-6 and monocyte chemoattractant protein-1 may act as predictor markers of renal function outcome in immunoglobulin A nephropathy. *Nephrology (Carlton)* 2009; 14: 613-620

30. Fornoni A, Ijaz A, Tejada T, Lenz O. Role of inflammation in diabetic nephropathy. *Curr Diabetes Rev* 2008; 4: 10-17

31. Thomas MC, Burns WC, Cooper ME. Tubular changes in early diabetic nephropathy. *Adv Chronic Kidney Dis* 2005; 12: 177-186

32. Abbate M, Zoja C, Remuzzi G. How does proteinuria cause progressive renal damage? *J Am Soc Nephrol* 2006; 17: 2974-2984

33. Zoja C, Garcia PB, Remuzzi G. The role of chemokines in progressive renal disease. *Front Biosci* 2009; 14: 1815-1822

34. Brinkman JW, de Zeeuw D, Duker JJ *et al*. Falsely low urinary albumin concentrations after prolonged frozen storage of urine samples. *Clin Chem* 2005; 51: 2181-2183

35. Brinkman JW, de Zeeuw D, Lambers Heerspink HJ *et al*. Apparent loss of urinary albumin during long-term frozen storage: HPLC vs immunonephelometry. *Clin Chem* 2007; 53: 1520-1526

36. Brinkman JW, de Zeeuw D, Gansevoort RT *et al*. Prolonged frozen storage of urine reduces the value of albuminuria for mortality prediction. *Clin Chem* 2007; 53: 153-154

37. Brinkman JW, Heerspink HL, de Zeeuw D, Gansevoort RT, Bakker SJ. Urinary pH affects albumin concentrations after prolonged frozen storage. *Nephrol Dial Transplant* 2007; 22: 3670

38. Chapman JR, O'Connell PJ, Nankivell BJ. Chronic renal allograft dysfunction. *J Am Soc Nephrol* 2005; 16: 3015-3026

39. Racusen LC. The Banff schema and differential diagnosis of allograft dysfunction. *Transplant Proc* 2004; 36: 753-754

40. Kasiske BL, Kalil RS, Lee HS, Rao KV. Histopathologic findings associated with a chronic, progressive decline in renal allograft function. *Kidney Int* 1991; 40: 514-524

41. Kasiske BL, Heim-Duthoy KL, Tortorice KL, Rao KV. The variable nature of chronic declines in renal allograft function. *Transplantation* 1991; 51: 330-334

42. Kasiske BL, Andany MA, Danielson B. A thirty percent chronic decline in inverse serum creatinine is an excellent predictor of late renal allograft failure. *Am J Kidney Dis* 2002; 39: 762-768

43. Kasiske BL. Proteinuria and other urinary biomarkers in kidney transplantation: why are we still waiting for godot? *Am J Kidney Dis* 2011; 57: 654-656

44. van Timmeren MM, Vaidya VS, van Ree RM *et al*. High urinary excretion of kidney injury molecule-1 is an independent predictor of graft loss in renal transplant recipients. *Transplantation* 2007; 84: 1625-1630

45. Schaub S, Mayr M, Honger G *et al*. Detection of subclinical tubular injury after renal transplantation: comparison of urine protein analysis with allograft histopathology. *Transplantation* 2007; 84: 104-112

46. Halimi JM, Buchler M, Al Najjar A *et al*. Urinary albumin excretion and the risk of graft loss and death in proteinuric and non-proteinuric renal transplant recipients. *Am J Transplant* 2007; 7: 618-625

47. Spoelstra-de Man AM, Brouwer CB, Stehouwer CD, Smulders YM. Rapid progression of albumin excretion is an independent predictor of cardiovascular mortality in patients with type 2 diabetes and microalbuminuria. *Diabetes Care* 2001; 24: 2097-2101

48. Brantsma AH, Bakker SJ, de Zeeuw D, de Jong PE, Gansevoort RT. Extended prognostic value of urinary albumin excretion for cardiovascular events. *J Am Soc Nephrol* 2008; 19: 1785-1791

49. Peters HP, Waanders F, Meijer E *et al*. High urinary excretion of kidney injury molecule-1 is an independent predictor of end-stage renal disease in patients with IgA nephropathy. *Nephrol Dial Transplant* 2011;

50. Branten AJ, du Buf-Vereijken PW, Klasen IS *et al*. Urinary excretion of beta2-microglobulin and IgG predict prognosis in idiopathic membranous nephropathy: a validation study. *J Am Soc Nephrol* 2005; 16: 169-174

51. Bolignano D, Lacquaniti A, Coppolino G *et al*. Neutrophil gelatinase-associated lipocalin (NGAL) and progression of chronic kidney disease. *Clin J Am Soc Nephrol* 2009; 4: 337-344

52. Hofstra JM, Deegens JK, Steenbergen EJ, Wetzels JF. Urinary excretion of fatty acid-binding proteins in idiopathic membranous nephropathy. *Nephrol Dial Transplant* 2008; 23: 3160-3165

53. Vaidya VS, Niewczas MA, Ficociello LH *et al*. Regression of microalbuminuria in type 1 diabetes is associated with lower levels of urinary tubular injury biomarkers, kidney injury molecule-1, and N-acetyl-beta-D-glucosaminidase. *Kidney Int* 2011; 79: 464-470

54. Kern EF, Erhard P, Sun W, Genuth S, Weiss MF. Early urinary markers of diabetic kidney disease: a nested case-control study from the Diabetes Control and Complications Trial (DCCT). *Am J Kidney Dis* 2010; 55: 824-834

55. Bolignano D, Lacquaniti A, Coppolino G *et al*. Neutrophil gelatinase-associated lipocalin as an early biomarker of nephropathy in diabetic patients. *Kidney Blood Press Res* 2009; 32: 91-98

56. Nielsen SE, Andersen S, Zdunek D, Hess G, Parving HH, Rossing P. Tubular markers do not predict the decline in glomerular filtration rate in type 1 diabetic patients with overt nephropathy. *Kidney Int* 2011; 79: 1113-1118

Nederlandse samenvatting

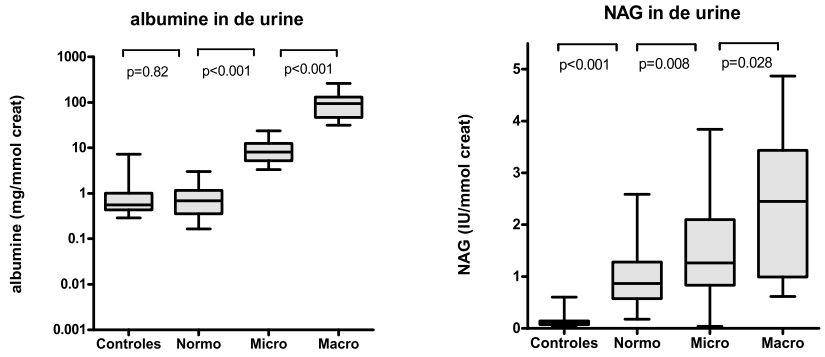
Het aantal mensen met een gestoorde nierfunctie is de afgelopen decennia sterk toegenomen. Een gestoorde nierfunctie betekent dat deze mensen een hoger risico hebben,om hart- en vaatziektes te krijgen,eindstadium nierfalen te ontwikkelen of om te overlijden. Mensen met een minder goede nierfunctie hebben hier aanvankelijk weinig klachten van, waardoor zij vaak pas laat medische hulp zoeken en daarmee pas in een laat stadium wordt ontdekt dat hun nierfunctie gestoord is. Screening op eiwitverlies in de urine (albuminurie) is momenteel de methode welke gebruikt wordt om patiënten met nierschade in een vroeg stadium op te sporen. Het belangrijkste doel van dit proefschrift was te onderzoeken of stoffen die in de urine uitgescheiden worden en schade aan de nier weergeven (toegevoegde) voorspellende waarde hebben boven de nu meest gebruikte schademarker, albumine uitscheiding in de urine. Deze nieuwe markers, zouden verscheidene toepassingen kunnen hebben. Naast dat ze zouden kunnen helpen om mensen met nierschade in een vroeg stadium op te sporen zouden ze ook gebruikt kunnen worden om te voorspellen bij welke patiënten de nierfunctie versneld achteruit zal gaan. Een derde toepassing zou kunnen zijn gelegen in het monitoren van de effecten van medicatie op de nier, waardoor bij een te hoge uitscheiding van de markers in de urine tijdens behandeling de medicatiedosering kan worden aangepast. Hierdoor zou toekomstige schade kunnen worden verminderd of voorkomen.

Hoofdstuk 1 gaat over het screenen van mensen op gestoorde nierfunctie. De diagnose chronische nierschade wordt bepaald door twee factoren: een verminderde nierfunctie uitgedrukt als een glomerulaire filtratiesnelheid kleiner dan 60 milliliter per minuut of uitscheiding van meer dan 30 mg albumine in de urine per dag. De glomerulaire filtratiesnelheid (GFR) is de belangrijkste maat voor nierfunctie. Het geeft het aantal milliliters bloed weer dat door de nier wordt gezuiverd per tijdseenheid. Hoe hoger deze glomerulaire filtratiesnelheid, des te meer bloed door de nier wordt

gezuiverd, en des te beter de nierfunctie is. Albumine is het eiwit dat het meest in het bloed voorkomt. Uitscheiding hiervan in de urine betekent dat of het filter waardoor bloed in de nier gefilterd wordt defect is, of dat de terugopname van albumine in de nierbuisjes (niertubuli) niet goed werkt. In hoofdstuk 1 wordt besproken dat deze twee variabelen, glomerulaire filtratiesnelheid en albumine uitscheiding in de urine, welke beide de diagnose chronische nierschade bepalen, onafhankelijk van elkaar kunnen voorspellen wie een grotere kans heeft verdere nierfunctiestoornissen te ontwikkelen. In de gezonde nier komen er nauwelijks eiwitten door het nierfilter en de eiwitten die er wel doorkomen worden vervolgens in de niertubuli weer heropgenomen, zodat uiteindelijk de urine vrijwel geen eiwitten bevat. Het is onbekend in welke mate de heropname van albumine plaatsvindt door de niertubuli. Wat wel vast staat is dat een beschadigde niertubulus albumine slecht opneemt. Het zou hierom goed kunnen zijn dat specifieke markers van schade van de niertubulus nog beter kunnen voorspellen wie nierschade heeft cq gaat ontwikkelen. Deze markers zouden bovendien voorspellende waarde kunnen toevoegen aan albumine uitscheiding in de urine in het voorspellen van klinische eindpunten, zoals het optreden van nierfunctie achteruitgang.

Om te kijken of deze tubulaire markers voorkomen bij mensen met een verhoogde albumine uitscheiding in de urine, hebben we in **hoofdstuk 2** bij 94 patiënten met suikerziekte (die door hun ziekte een hoog risico lopen om nierschade te ontwikkelen) onderzocht of deze markers van nierschade in de urine aanwezig zijn. Ook hebben we ter vergelijking in urine van een controlegroep bestaande uit 45 gezonde mensen deze markers gemeten. Het bleek dat de schademarkers van de niertubuli al verhoogd waren bij patiënten met suikerziekte in vergelijking met de waarden die werden gemeten bij de controlegroep. Dit gold zelfs voor de patiënten met suikerziekte die nog een normale nierfunctie en normale uitscheiding van albumine in de urine hadden (figuur 1). Deze resultaten suggereren dat de markers al in een zeer vroeg stadium nierschade weerspiegelen, waardoor

ze van hulp zouden kunnen zijn om nierpatiënten al in een heel vroeg stadium van de nierziekte op te sporen. Verder is onderzocht hoe de schademarkers zich verhouden tot de uitscheiding van albumine in de urine en hoe ze zich verhouden tot de nierfunctie (GFR). Het bleek dat de uitscheiding van bijna alle markers toeneemt naarmate er meer albumine uitscheiding in de urine is. Figuur 1 toont dit voor bijvoorbeeld albumine en NAG uitscheiding. Ook neemt de uitscheiding van deze markers toe naarmate de nierfunctie afneemt. Op grond hiervan kan geconcludeerd worden dat de onderzochte markers samenhangen met nierschade en dat het waarschijnlijk is dat zij het ontstaan van nierschade al in een vroeg stadium kunnen voorspellen.



Figuur 1: Concentratie van verschillende schademarkers van de nier in de urine. Gezonde controles zijn links afgebeeld; Normo betekent een normale uitscheiding van albumine in de urine; Micro betekent een licht verhoogde uitscheiding van albumine in de urine; Macro houdt een fors verhoogde uitscheiding van albumine in de urine in. Een p-waarde kleiner dan 0.05 betekent dat de groepen statistisch significant van elkaar verschillen.

Op dit moment is albumine in de urine een belangrijk middel om de mate van nierschade te definiëren.Het is dus belangrijk dit betrouwbaar te kunnen meten. In veel studies wordt gebruikt gemaakt van urinemonsters die ingevroren zijn geweest. Het is bekend dat door invriezen de concentratie van albumine in de urine omlaag gaat en er meer variatie ontstaat. In **hoofdstuk 3** is onderzocht of het aanlogen van urine (toevoegen van een vloeistof die de zuurgraad van urine verminderd) voordat de urine ingevroren

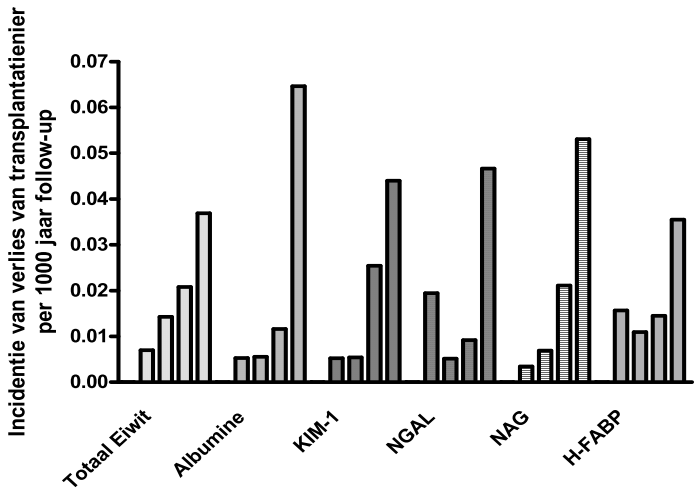
wordt voorkomt dat de meetbare albumineconcentratie verandert door het zogenaamde vries-dooi effect. Hiervoor zijn urinemonsters van patiënten met suikerziekte verzameld en ingevroren. De albumine concentratie werd gemeten in het verse monster en op verschillende tijdspunten na invriezen. De monsters die 1 jaar werden bewaard zijn zowel bij -20º C en bij -80º C bewaard en vervolgens gemeten. Uit dit onderzoek is gebleken dat, als monsters 1 jaar bij -20 ºC worden bewaard, aanlogen van de urinemonsters de afname van de albumineconcentratie en de toename in variabiliteit vermindert in vergelijking met de monsters die niet voorbehandeld worden. Verder werden geen verschillen aangetroffen tussen niet aangeloogde monsters die 1 jaar bij -80º C waren bewaard en de aangeloogde monsters die bij -20º C waren bewaard. Dit maakt dat het aanlogen van urine en het vervolgens bewaren bij -20º C een geschikt alternatief is voor bevroren opslag bij -80º C.

Aangezien we voor onze vervolgstudies niet alleen albumine, maar ook een aantal schademarkers van de nier wilden meten, en er erg weinig bekend is over de effecten van bevroren opslag op de concentratie en variabiliteit van deze schademarkers, hebben we in **hoofdstuk 4** onderzocht wat de effecten zijn van bevroren opslag op de concentratie van een panel van verschillende schademarkers van de nier. Een dergelijk onderzoek is van belang omdat eventuele vrieseffecten, zoals daling van de meetbare concentratie van deze schademarkers of toename in variabiliteit, ten onrechte het beeld zouden kunnen doen ontstaan dat schademarkers niet goed schade weerspiegelen of de nierprognose niet goed voorspellen. In de studie die hier wordt beschreven hebben we urinemonsters van 95 patiënten met suikerziekte wederom 1 jaar opgeslagen. Hierbij hebben we verschillende bewaarprotocollen toegepast. Zo hebben we urines voor het invriezen aangeloogd, hebben we proteaseremmers toegevoegd en hebben we na het ontdooien de monsters aangeloogd. Tevens zijn de monsters in twee verschillende vriezers zowel bij -20º C als bij -80º C opgeslagen. Om te onderzoeken of een eventuele afname van de marker concentraties groter

wordt naarmate een monster langer wordt bewaard, hebben we monsters eerst vers gemeten (in dezelfde meetsessie twee keer, en nog een keer direct na de eerste meetsessie), na 1 week bevroren opslag, na 6 maanden en na 1 jaar. Hieruit bleek dat behalve cystatine C alle markers na langere opslag in concentratie afnamen en een toename in variabiliteit toonden na 1 jaar bevroren opslag. Daarnaast werd duidelijk dat verschillende bewaarprotocollen (zoals voor of na invriezen aanlogen of proteaseremmers toevoegen) deze effecten niet konden voorkomen. Ten derde is onderzocht wat de relatie was tussen vers gemeten concentraties en de na bevroren opslag gemeten concentraties. Deze relaties bleken over het algemeen redelijk goed. Op grond van deze gegevens concluderen we dat deze markers het best gemeten kunnen worden in verse urine monsters. Indien dit niet mogelijk is dan kunnen urine monsters worden gebruikt die bevroren opgeslagen zijn geweest. Echter, er kan dan sprake zijn van een vrieseffect (afname in concentratie en toename in variabiliteit). Als er dan geen relatie wordt gevonden tussen een marker die gemeten is in urine monsters die ingevroren zijn geweest en er geen relatie wordt gevonden tussen de marker concentratie en het onderzochte eindpunt, dan hoeft dit dus niet noodzakelijkerwijs te betekenen dat deze relatie er niet is. Het zou ook kunnen zijn dat deze relatie vertroebeld is door een vries-dooi effect.

Zoals eerder in hoofdstuk 2 is besproken, kunnen de in dit proefschrift in de urine gemeten schademarkers al verhoogd zijn voor dat één van de conventionele maten voor nierschade (albumine en eGFR) afwijkend is. Daarom is in **hoofdstuk 5** onderzocht of deze nieuwe markers van schade van de nier bij patiënten die een niertransplantatie hebben gekregen voorspellen of die nier door chronische afstoting verloren gaat. Dit is onderzocht met behulp van urinemonsters die bevroren opgeslagen zijn geweest. Voor dit onderzoek is een groep van 606 patiënten onderzocht, die hiervoor 4.7 jaar gevolgd zijn. Van deze 606 patiënten verloren 42 de functie van hun transplantatienier. Tevens is onderzocht of de genoemde schademarkers gedeeltelijke achteruitgang in nierfunctie voorspellen. Uit de

uitkomsten is af te leiden dat alle onderzochte markers verlies van de transplantaatnier voorspellen, maar dat zij dit niet beter doen dan de nu in de klinische praktijk veel gebruikte marker totaal eiwit. Opvallend is echter dat de uitscheiding van albumine in de urine verlies van de transplantaatnier beter voorspelde dan de onderzochte schademarkers en ook beter dan het nu veel gebruikte totaal eiwit in de urine. Als deelnemers in vier groepen met oplopende concentratie schademarkers worden ingedeeld, treedt het voorkomen van het verlies van functie van de transplantatienier meer op naarmate de concentratie van deze schademarkers hoger is (figuur 2). Omdat maar een klein deel van alle onderzochte mensen volledig verlies van functie van hun niertransplantaat hadden, is ook gekeken naar de mate van nierfunctie verlies. Twee van de vier tubulaire schademarkers voorspelden ook nierfunctie achteruitgang. Albumine in de urine voorspelde dit echter ook, en opnieuw beter dan het nu in de klinische praktijk veel gebruikte totaal eiwit in de urine. Hieruit kan geconcludeerd worden dat het in de klinische praktijk het beste is om bij deze patiëntengroep in de urine albumine te meten, omdat het beide eindpunten beter voorspelt dan totaal eiwit en beter dan de onderzochte nieuwe nierschade markers.



Figuur 2: de verticale as toont de incidentie van verlies van transplantatienier. Incidentie is het voorkomen van een gebeurtenis per bepaalde tijdsduur vervolgen. Op de x-as zijn alle onderzochte schademarkers opgedeeld in kwartielen van hogere concentratie.

In hoofdstuk 2 en hoofdstuk 5 is concentratie van tubulaire schademarkers onderzocht bij patiënten met een chronische nierziekte. Er is echter ook weinig bekend over de waarde van deze markers bij mensen uit de algemene bevolking om te voorspellen of zij progressief nierschade gaan ontwikkelen . Het is van groot belang hier inzicht in te krijgen, omdat het vroeg opsporen van mensen die hoog risico lopen om nierschade te ontwikkelen, de mogelijkheid biedt in een vroeg stadium te starten met behandeling. Hierdoor kunnen eventuele gevolgen van nierschade worden voorkomen of verminderd. Daarom is in **hoofdstuk 6** onderzocht of de verschillende schademarkers het ontstaan van nierschade (gemeten als toename van albumine uitscheiding in de urine) na het meerdere jaren vervolgen kunnen voorspellen. Voor dit onderzoek is gebruik gemaakt van deelnemers aan de PREVEND studie De PREVEND studie bestaat uit 8592 mensen, die gemiddeld meer dan 9 jaar gevolgd zijn. Uit deze groep zijn diegenen geselecteerd met de 20% meeste toename in albumine uitscheiding in de urine. Dit leverde 183 mensen op. Deze mensen zijn gekoppeld aan 2x zoveel controlepersonen, die wat betreft leeftijd, geslacht en hoeveelheid albumine in de urine aan de start van de studie overeenkomen. In de urine van al deze mensen (549 in totaal) zijn schademarkers van de nier gemeten. Het bleek dat deelnemers die in de loop der jaren een forse toename in albumine uitscheiding hebben, in vergelijking met controlepersonen, een hogere urine uitscheiding van schademarkers van het nierfilter (glomerulus) hebben, maar juist lagere concentraties van schademarkers van de niertubulus. Hieruit kan worden geconcludeerd dat verergering of het ontstaan van nierschade meer samenhangt met tekenen van een kapot nierfilter (glomerulus) en minder met een slecht functionerende niertubulus. Dit betekent dat albumine uitscheiding in de urine als gevolg van een tubulusopnameprobleem relatief onschuldig is, terwijl albumine in de urine als gevolg van een lek nierfilter meer problematisch lijkt te zijn.

In zowel hoofdstuk 5 als 6 is onderzocht of schademarkers kunnen voorspellen wie nierschade zal gaan ontwikkelen. Deze markers kunnen echter niet alleen gebruikt worden om het optreden van schade te voorspellen, maar bijvoorbeeld ook om te onderzoeken of een medicament een nierbeschermend effect heeft. Ook zou het meten van deze markers mogelijkheden kunnen bieden om de dosering van een medicament individueel aan te passen. Als bijvoorbeeld een patiënt een hoge uitscheiding van schademarkers in de urine heeft, dan kan de dosering van een nierbeschermend medicament aangepast worden totdat de uitscheiding van schademarkers normaal is. Hierdoor kan het medicament voorgeschreven worden in een dosering waarmee achteruitgang van nierfunctie het meest optimaal voorkomen kan worden. In **hoofdstuk 7** is in dit kader onderzocht wat het effect is van een lage zoutinname en het geven van een angiotensine II receptor blokker (ARB). Dit laatste is een medicijn dat bij patiënten met een nierziekte wordt voorgeschreven om achteruitgang van nierfunctie te voorkomen. Voor dit onderzoek zijn, in urine van 52 patiënten met een chronische nierziekte, verschillende schademarkers van de nier gemeten. Patiënten werden behandeld in vier periodes van zes weken in willekeurige volgorde (normale zoutinname, normale zoutinname+ ARB, lage zoutinname en lage zoutinname+ARB). Aan het einde van iedere zes weken durende periode is urine verzameld. Hierin zijn de schademarkers bepaald. Uit de verkregen gegevens kan geconcludeerd worden dat verlaging van de zoutinname een grotere afname van nierschademarkers met zich mee bracht dan het geven van een ARB. Verder is onderzocht of het nastreven van een strengere behandeldoelstelling voor de eiwituitscheiding in de urine (dat wil zeggen het toevoegen van nierbeschermende maatregelen tot een totale eiwituitscheiding van minder dan 0.3 gram per dag wordt bereikt) gepaard gaat met lagere uitscheiding van de onderzochte schademarkers. Bij de start van de studie hadden alle patiënten een eiwituitscheiding in de urine van meer dan 1 gram per dag..Het bleek dat een verlaging van de eiwituitscheiding tot kleiner dan 0.3 gram per dag door lagere zoutinname of toevoeging van een ARB weliswaar

gepaard ging met lagere uitscheiding van schademarkers, maar dat deze afname niet proportioneel is aan de afname in eiwituitscheiding in de urine. Dit suggereert dat er nog steeds schade is, zelfs als de strengere behandeldoelstelling wordt gehaald.

Conclusie

Dit proefschrift toont aan dat er voor het meten van de onderzochte schademarkers van de nier diverse nieuwe toepassingen kunnen zijn. Zo kunnen de markers van waarde zijn bij het opsporen van patiënten met nierschade die door de conventionele methodes nog niet worden opgespoord (hoofdstuk 2). Ook kunnen de markers gebruikt worden om de prognose van nierschade te voorspellen, zoals in hoofdstuk 5 en 6. Verder zouden ze gebruikt kunnen worden om bij individuele patiënten nierbeschermende medicijnen optimaal te doseren. Dit zijn veelbelovende ontwikkelingen. De studies en uitkomsten in dit proefschrift zullen echter eerst moeten worden bevestigd in andere onderzoeken voordat ze in de klinische praktijk kunnen worden ingezet. Tot op heden is het nog niet volledig duidelijk of deze schademarkers beter zijn dan, of voorspellende waarde kunnen toevoegen aan de op dit moment meest gebruikte marker voor chronische nierziekte, namelijk albumine uitscheiding in de urine. Om dit verder te onderzoeken zijn grote studies nodig, in goed vastgestelde patiëntengroepen waarbij het liefst verse urine monsters worden gebruikt, of zorgvuldige bewaarprotocollen worden gebruikt om de urinemonsters in te vriezen. Zulke studies kunnen dan duidelijk maken of bepaalde nieuwe schademarkers van de nier (of mogelijk combinaties daarvan) de voorspelling van het verloop van nierziekte kunnen verbeteren. Totdat zulke studies zijn verricht blijft de belangrijkste schademarker die gemeten kan worden om de prognose van nierpatiënten te bepalen albumine.

Dankwoord

Het feit dat ik nu mijn dankwoord aan het schrijven ben, betekent dat mijn boekje bijna af is. Het zou goed kunnen dat dit het enige deel van het boekje is dat echt iedereen leest. Dit wordt ongetwijfeld een lange lijst met namen van mensen die mij allemaal geholpen/bijgestaan hebben. Mocht ik onverhoopt toch nog iemand vergeten zijn, bij deze op voorhand mijn oprechte excuses en natuurlijk ook mijn dank voor de hulp.

Als eerste wil ik graag alle patiënten en PREVEND deelnemers bedanken die hebben deelgenomen aan de studies in dit proefschrift. Zonder jullie was dit proefschrift er niet geweest. Ook alle collega-onderzoekers, vrienden, kennissen en vrienden van kennissen van vrienden die allemaal belangeloos bloed hebben afgestaan of urine hebben verzameld: bedankt!

Verder wil ik graag mijn eerste co-promotor bedanken. Dr R.T. Gansevoort, beste Ron. Ik wil je graag bedanken voor je kritische blik, enthousiasme en overzicht. Na drie jaar samenwerking verras je me nog steeds met je frisse kijk op mijn data en artikelen (en layout). Ik verbaasde me meer dan eens hoe je soms met minimale tekstuele wijzigingen (ook wel ’s wat meer trouwens) een manuscript flink verbeterde. Zonder jouw hulp en frequente begeleiding was dit proefschrift zeker niet geworden wat het nu is.

Verder wil ik uiteraard mijn promotor, professor P.E. de Jong hartelijk bedanken. Beste Paul, ik heb bewondering voor je enthousiasme en oog voor detail. Meerdere keren verbaasde je me met de snelheid waarmee je mijn manuscripten van opbouwende kritiek voorzag en scherpte waarmee je de inhoud doornam (een onlogisch getal in een grote tabel ontgaat jou niet!).

Verder wil ik ook mijn tweede en derde co-promotores bedanken. Dr S.J.L. Bakker, beste Stephan, dank voor je statistische en inhoudelijke input. Dr W. van Oeveren, beste Wim, wij hebben samen heel wat vrijdagmiddagen tot in de avond op het lab gezeten om onze veelvoud aan (te?) strak geplande

ELISA's tot een goed einde te brengen. Ik heb veel geleerd van je praktische insteek en laboratoriumkennis. Zonder dit alles was dit boekje niet mogelijk geweest.

Graag wil ik de leden van de beoordelingscommissie prof R.O.B. Gans, prof J.F.M. Wetzels en prof H. van Goor bedanken voor het beoordelen van mijn proefschrift.

Bij een proefschrift is de praktische ondersteuning erg belangrijk. Beste Winnie, het was soms een hele klus om een afspraak met al m'n begeleiders op hetzelfde moment op dezelfde kamer te plannen. Dank voor je hulp en natuurlijk de gesprekjes op de BRUG! Ook Jan Roggeveld, jij stond altijd klaar om samples voor me te meten. Ook was je altijd weer bereid samen op speurtocht te gaan in het serumarchief om bepaalde monsters te zoeken. Hetzelfde geldt ook voor Bettine: ook jij stond altijd klaar om ELISA tips te geven en monsters te meten.

Een onmisbaar onderdeel van een promotietraject zijn de collega AIO's. Ik heb genoten van de goede sfeer, de vrimibo's en de vanzelfsprekendheid waarmee iedereen elkaar hier helpt.
Esther, heerlijk was het om je gevatte opmerkingen te horen (over zowel werk als privé). Beste Nynke, ik heb gedurende mijn begin veel van je statistische kennis mogen profiteren. Dank voor je vanzelfsprekende interesse! Verder ook bedankt voor je gezelligheid en natuurlijk ook voor je kooktips (misschien doe ik er ooit nog wat mee?).
Beste Marije, roommie, ook jij bedankt voor je statistical coaching toen ik volstrekt onwetend op deze afdeling begon. Sorry voor alle keren dat ik je uit je concentratie trok voor een vraag en je vervolgens - geheel Marije style - weer opnieuw moest 'opstarten'. En natuurlijk ook bedankt voor de gezelligheid (dankzij jou bleef ik op de hoogte van de celebrity wereld) en de vele directe gevraagde en ongevraagde adviezen.

Beste Lieneke, ik heb genoten van je sprankelende vrolijkheid, je interesse en je theaterkwaliteiten. Gelukkig wist je, als ik weer onduidelijke frasen uitkraamde, precies wat ik bedoelde en op welke data wat precies was. Je hebt je ontzettend snel ontwikkeld tot een grote SPSS specialist. Met je boekje zal het daarom zeker goed komen!
Beste Wendy, allereerst natuurlijk dank voor al je hulp bij het zien van alle diabetes patiënten in Zwolle, de metingen en het opzetten van de database. Verder was je een trouwe bezoekster van onze vrimibo's, dank ook voor de gezelligheid daar!
Beste Hanneke, jammer genoeg zijn we maar kort roommies geweest. Ik ken niemand die zo geconcentreerd kan werken. Dank ook voor je altijd gezellige aanwezigheid en je klinische blik op de zaak! Beste Lucia, ook jij van harte bedankt voor de vele gesprekjes, (mode- en haardracht-) adviezen en gezelligheid! Debbie, als roommie hoor jij hier ook. Dank voor je frisse kijk en heel veel succes met onderzoek doen! Edwin, chef, we hebben maar kort een werkkamer gedeeld, maar je hebt mijn woordenschat aan studentikoze uitdrukkingen flink uitgebreid!

Over naar de BRUG. Else, aan jouw stroom gevatte opmerkingen (op onze eerste ontmoeting: "Ferdau, is dat niet een anagram van fraude?") komt nooit een eind. Ik heb genoten van je interesse en gezelschap op en buiten het werk! Ook super dat je mijn paranimf wilt zijn. Hilde, jij kon wel eens degene zijn naar wie ik tijdens mijn onderzoeksperiode de meeste mailtjes heb gestuurd. Jij ook bedankt voor je gezelligheid, interesse, trouwe aanwezigheid op de vrimibo en ontbijtsessies. Oosterpoort!
Steef, jongen, ook jij steekt je mening niet onder stoelen of banken. Bedankt voor de scherp geformuleerde adviezen en ik hoop nog vaak samen op wintersport te kunnen gaan.
Arjan, ook jouw vrolijke aanwezigheid zal ik gaan missen! Tsjitske, op jouw nuchtere kijk en enthousiasme kon ik altijd rekenen. Ik had het idee dat we altijd als één van de laatsten op borrels overbleven. Dank voor je gezelligheid!

Dorien, één keer heb ik mogen ervaren wat jij meerdere malen per week doet. Respect! Maar ik houd het bij één springklasje… Bedankt voor je leuke gezelschap tijdens het werk en congressen! Jan, de man die op z'n rug kan zitten. Jij ook bedankt voor je mooie verhalen, gedetailleerde kennis van de lokale fauna waar dan ook en gezelligheid! Maartje, ook jou bedank ik voor je enthousiasme, prettige samenwerking en gezelligheid uiteraard! Ik ben blij dat we nu weer collega's te zijn! Solmaz, bedankt voor je vrolijkheid, gezelligheid en lessen Farsi (hawo goebè!). Merel, waarschijnlijk ben jij de volgende. Dank voor je plezierige aanwezigheid en veel succes in Amsterdam!
Michel, jij bent een vrolijke aanvulling op het stel. Jammer dat we maar kort tegelijk in het UMCG werkten. Goed dat er wat mannelijke input is in deze door dames gedomineerde onderzoeksclub!

Verder wil ik ook alle andere AIO's en inmiddels gepromoveerden die ik korter of langer heb gekend bedanken voor de gezelligheid op en buiten het werk. Mariam, bedankt voor je geduldige uitleg als ik iets expirimenteels weer volstrekt niet begreep. En natuurlijk voor de gezelligheid. Alaa, Anna, Astrid, Azideh, Carolien, Eelke, Folkert, Femke, Guiseppe, Gemma, Heleen, Jeffrey, Jelena, Kevin, Kiran, Laura, Leendert, Miriam, Pramod, Rutger, Saleh, Saritha, Shanti, Willem, Ineke en Janneke, bedankt!

Ook de doktersassistentes van PREVEND mogen niet onbenoemd blijven! Martha, Annemarie, Annet, Annie en Martha, zonder jullie inzet bij de dataverzameling van PREVEND had mijn boekje er niet hetzelfde uit gezien. Dank ook voor de vele koffiemomenten toen we nog op dezelfde gang zaten en de huishoudelijke adviezen!

De post-doc's die mij op verschillende manieren hebben geholpen wil ik ook graag hartelijk bedankten. Hiddo, bedankt voor de prettige samenwerking en de vele statistische adviezen! Martin, jij ook bedankt voor het leuke COX-2 stuk! Eefje, fijn dat ik mocht langslopen voor moeilijke statistische issues.

Ook mijn nieuwe collega's in het Martini ziekenhuis wil ik bedanken voor de warme ontvangst!

Vrienden mogen absoluut niet onbenoemd blijven in mijn dankwoord! Een promotietraject is alleen vol te houden met goede vrienden die voor afleiding zorgen.
Aljar, bedankt dat je m'n paranimf wilde zijn. Ik zou zeggen, Romeo Delta. Verder is een vakantiedag ook niet compleet zonder jouw commentaar en 'probleempjes' (weten we nu al wat de kooktemperatuur is van kwik in vacuum?). Hilbert, biertjes drinken, vakantievieren en weekendjes-weg zijn zonder jouw commentaar en nuchterheid niet hetzelfde. Hopelijk volgen nog vele air- sea- en land miles samen. Cees, glad you're back, hoewel een vast logeeradres in Berlijn ook verleidelijk is. Gerda, jij ook bedankt voor je frisse kijk op zaken en vriendschap. En natuurlijk voor het lay-outen van dit prachtige proefschrift! Liesbeth, ik geniet iedere keer weer van een nieuwe discussie of iets psychiatrisch is of normaal. Bert, super dat we straks weer in hetzelfde ziekenhuis werken. Dank voor je gezelligheid en je linkse kritische geluid! Rogier, fijn dat je weer terug bent. Wat mij betreft gaan we nog heel wat baantjes trekken. Karin, leuk dat we elkaar weer veel zien in het Martini!

Lieve familie, ook jullie zullen hier niet ontbreken. Sietske, Jarno en Julia, Marrit en Tim, bedankt voor de interesse en steun gedurende dit hele traject. Papa en mama, jullie hebben mij altijd gestimuleerd en ondersteund in de dingen die ik graag wilde doen. Zonder jullie aanhoudende support had ik dit nu niet bereikt!